Analytical studies on the N-dealkylation of drug molecules by Cunninghamella sp.

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ANALYTICAL STUDIES ON THE N-DEALKYLATION OF DRUG MOLECULES BY *CUNNINGHAMELLA* SP.

THESIS

Submitted by Mark Gibson, B.Pharm., M.P.S.,
for the degree of Doctor of Philosophy
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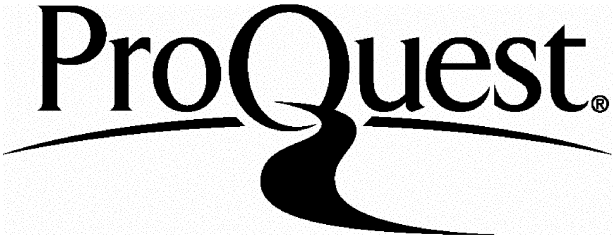
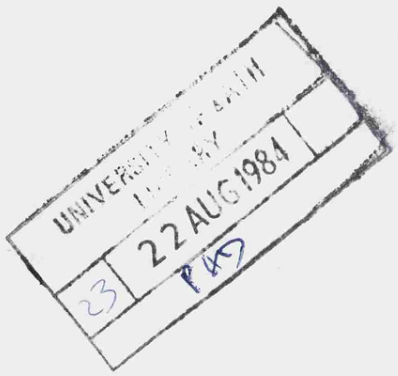
This research has been carried out in the School of Pharmacy and Pharmacology of the University of Bath under the supervision of C.J.Soper, B.Pharm., M.Sc., Ph.D., M.P.S. and Professor R.T. Parfitt, B.Pharm., Ph.D., F.P.S., Ch.Chem., F.R.I.C.




UNIVERSITY OF DURHAM
22 AUG 1984

A generalist starts by knowing a little about
a lot. With the course of time he comes to know
less and less about more and more until he
finally knows nothing about everything.
A specialist initially knows quite a lot about
quite a little. Gradually he comes to know
more and more about less and less until he
finally knows everything about nothing

Lord Elton

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Dedication

This thesis is dedicated to my parents for their unfailing support and encouragement of all my endeavours.

SUMMARY

In the Introduction the importance of N-dealkylation in pharmaceutical studies is summarised and the scope and potential advantages of microbial transformations in effecting N-dealkylation are examined. Possible applications of chromatographic and spectroscopic techniques to the study of microbial transformations are discussed.

Chapter 2 describes the development of quantitative HPLC techniques for the determination of a model substrate codeine, and its N-demethylated transformation product, norcodeine, from microbial transformation mixtures. Extraction procedures applicable to 7 litre fermenter cultures are also investigated.

The effect of carbon source and substrate concentration on the growth and N-demethylation of codeine by *Cunninghamella* sp. are investigated in Chapter 3.

Chapter 4 describes the development of procedures for the preparation of a cell-free extract from *Cunninghamella* sp. with optimal N-demethylase activity. Reaction conditions for N-demethylation of codeine by cell-free extracts of *C. bainieri* are optimised in terms of substrate concentration and co-factor requirements.

Kinetic data for the N-demethylation of codeine and codeine

N-oxide by *C. bainieri* are presented in Chapter 5. The effect of selected inducers and specific enzyme inhibitors is also examined. A reaction mechanism for the N-demethylation of codeine by cell-free extracts of *C. bainieri* is proposed.

Chapter 6 describes the development and application of a ^{13}C NMR spectroscopic technique to detect the possible intermediates in the N-demethylation of codeine by cell-free extracts of *C. bainieri*, and provides further evidence in support of the proposed reaction mechanism.

In Chapter 7 a HPLC technique is employed to estimate the lipid solubility of a selected series of 1,4-benzodiazepines from retention data. The dependence on substrate lipophilicity, of N-dealkylation of these compounds by *C. bainieri*, is examined.

The data are considered with reference to current microbial transformation literature. The proposed mechanism of microbial N-demethylation is compared to mammalian transformations of similar substrates and suggestions for future study are emphasised.

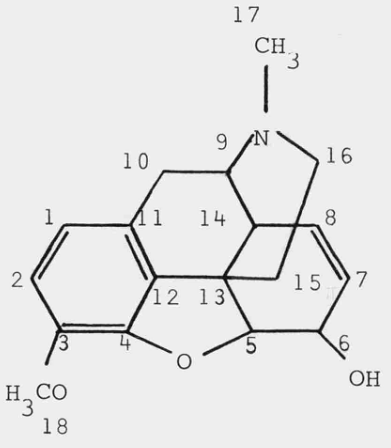
Abbreviations and Symbols

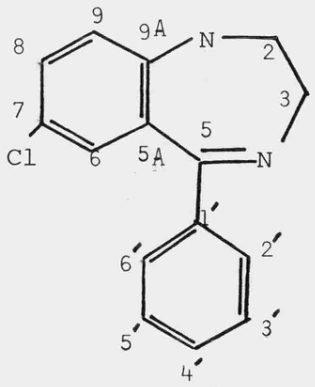
$A_{10\%}$	Symmetry factor
B.S.A.	Bovine Serum Albumin
^{13}C	Carbon-13
C.O.M.	Proton Noise Decoupling
C.W.	Continuous wave
ε	porosity
F.T.	Fourier Transformation
F.I.D.	Free Induction Decay
G.C.	Gas Chromatography
G.L.C.	Gas Liquid Chromatography
H.E.T.P.	Height Equivalent to a Theoretical Plate (H)
H P L C.	High pressure or High Performance Liquid Chromatography
H.P.L.S.C.	High Pressure Liquid-Solid Chromatography
h	Reduced plate height
I.R.	Infra-red
I.D. or i.d.	Internal diameter
κ	kappa or column capacity ratio
K_M	Michaelis constant
NADPH	Reduced Nicotinamide adenine dinucleotide-phosphate
N.O.E.	Nuclear Overhauser Effect
N.M.R. or nmr	Nuclear Magnetic Resonance
N	Theoretical plate number
O.D.S.	Octadecylsilane
OFR	Off-resonance decoupling
P.F.T	Pulse Fourier Transformation

ppm	parts per million
P	Partition Coefficient
Rs	Resolution
S/N	Signal to Noise ratio
t_R	Retention time
TMS	Tetramethylsilane
T.L.C.	Thin Layer Chromatography
U.V.	Ultra-violet
v	Reduced velocity
$\underline{V}$ or V_{max}	Maximum velocity

The numbering system used for ^{1}H and ^{13}C NMR spectral assignments for

(A) Codeine and related compounds (B) 1,4-benzodiazepines





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ORIGIN AND SCOPE

This project arose out of difficulties experienced by medicinal chemists in effecting N-dealkylation reactions by direct chemical means to prepare useful drug intermediates. Previous workers at Bath envisaged that microbial N-dealkylation procedures may provide a safer and more efficient alternative to chemical methods. Subsequently, they developed a microbial transformation system in which several species of the fungus *Cunninghamella* demonstrated the ability to N-demethylate codeine and a wide variety of drug molecules.

The studies reported in this thesis are concerned with further developments in this field of research. It was hoped that analytical techniques would be developed to enable a more detailed examination of the transformation processes involved. At the same time, it would be necessary to prepare from the micro-organisms cell-free extracts which maintain high N-dealkylation activity. This would enable the N-dealkylase enzyme(s) to be characterised. Of particular interest would be the elucidation of the mechanism of microbial N-dealkylation of the model substrate, codeine, since this would facilitate optimisation of the reaction. It was also intended to extend these studies to investigate alternative transformation substrates and transformation systems.

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

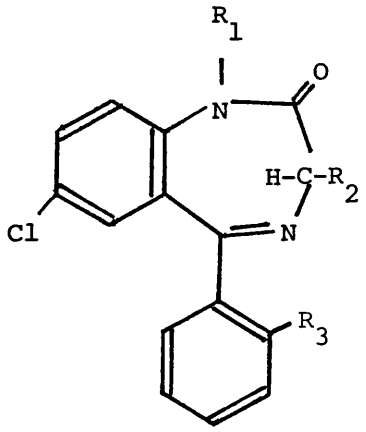
1.1 The pharmaceutical importance of N-dealkylation of drug molecules

N-alkyl functions are present in a wide variety of drug molecules, usually in saturated cyclic structures or alkylamine chains. In the design of a new drug it is often required to remove the N-alkyl function from the parent drug to form the nor-intermediate, and replace it with different N-substituents. This can be done to establish relationships between the chemical structures of a series of drugs and pharmacological activity. With this acquired knowledge it is possible to predict and design more selective products. This is demonstrated by the classification of the 1,4-benzodiazepine group of anxiolytics where studies by Sternbach *et al.*[1,2] demonstrated that these compounds were biologically active, provided that they possessed the 1,4-benzodiazepine ring system with both 7-chloro and 5-phenyl groups. The data in Table 1.1 illustrates how the modified N-substituent significantly affects the plasma half-life of the drug, and its presumed duration of action[3].

1.2 Chemical methods of N-dealkylation

The N-dealkylation of tertiary amines has traditionally been effected by the Von Braun reaction[4] employing cyanogen bromide. This reagent is extremely toxic, and by this procedure low or variable yields of the N-dealkylated product are often obtained[5],

Table 1.1. Effect of N-substituent variation on the classification of benzodiazepines according to duration of action.



Drug	R_3	R_2	R_1	Half-life* (hours)	Class of drug
Oxazepam	OH	H	-H	5-15	Short acting
Diazepam	H	H	$-CH_3$	26-53	Moderate long-acting
Prazepam	H	H	$-CH_2$-cyclopropyl	30-100	Long acting
Flurazepam	H	F	$-CH_2CH_2N(Et)_2$	40-200	Very long acting

* Plasma half-life in normal humans.

especially when complex alkaloidal molecules such as codeine are N-demethylated by this technique[6].

Subsequently, the Von Braun procedure has been superseded by alternative methods of N-dealkylation, in particular, those utilising chloroformate esters. These often improve selectivity and produce cleaner reaction products[7]. Chloroformates react with tertiary amines to give carbamates, and these are hydrolysed to give the corresponding secondary amine[5].

$$R_1R_2N\text{-}CH_3 \xrightarrow{ClCO_2R} R_1R_2NCO_2R \xrightarrow{\text{Hydrolysis}} R_1R_2NH$$

However, studies by Gadamer and Knoch on the effect of ethyl chloroformate on a variety of cyclic tertiary amines found that the N-methyl function of alkaloids such as morphine and tropine were not cleaved[8]. Phenylchloroformate[5] and vinylchloroformate[9] have been found superior to ethyl chloroformate in the cleavage of tertiary amines. More recently 2,2,2-trichloroethylchloroformate[10] and its derivatives have found wide application as protective groups to give carbamate intermediates. These are easily hydrolysed by treatment with zinc and acetic acid. Although N-demethylation with chloroformates may give satisfactory yields in many cases[5,9,11] they are all potent lachrymators and potentially hazardous reagents. Other chemical methods of N-dealkylation have been tried with limited success. For example, diethylazocarboxylate[12], and a photochemical procedure in the presence of oxygen[13]. Clearly there is a requirement for an alternative

N-dealkylation technique to improve product yields,and to eliminate the hazardous and toxic reagents used in existing chemical methods.

1.3 The scope and potential advantages of microbial transformations

A microbial transformation can be described as a simple, specific chemical modification of a substrate, effected by microbial enzymes, to produce a closely related product. In the case of N-dealkylation, the N-alkyl group is cleaved from the parent structure to leave an N-H bond.

Since the earliest microbial transformation reported by Pasteur[14] in 1864, the production of vinegar from ethanol, microbial transformation studies have progressed considerably. A wide range of substrates have been transformed, and a variety of reaction types have been effected by microorganisms[15]. These include oxidation, reduction, hydrolysis, dehydration, condensation and isomerisation reactions, and also the formation of new carbon-carbon or hetero-atom bonds[15]. It has been estimated that there is probably an enzyme catalysed equivalent for every type of chemical catalysed reaction[16]. Furthermore, microbial transformations offer a potential alternative to existing chemical methods of N-dealkylation.

There are a number of potential benefits of using microbial methods to synthesise drugs, as opposed to chemical methods. Primarily, microbial transformations may give rise to increased and more consistent yields. They are usually specific with respect to a particular substrate or a reaction type[16], and some reactions that are not chemically possible may be mediated by microorganisms[15]. It may even be possible to achieve several coupled reactions in one transformation step[15]. In addition, microbial transformations are normally conducted under mild conditions, in aqueous media, with cheap raw materials as growth media, so they are generally economical. Especially as the unreacted starting material may be recovered and recycled[15]. The mild conditions may also ensure that complex medicinal compounds are not decomposed in the transformation mixture, especially if the compound is acid, base or heat labile[17].

However, some disadvantages that may be incurred with microbial transformations should also be considered. These include the necessity to operate under sterile conditions, the high energy consumed in sterilising procedures, and the expense of recovering and purifying the transformation product[15].

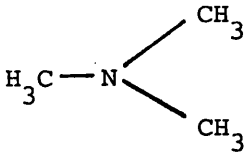
1.4 Studies on the microbial N-dealkylation of drug molecules

Previous workers at Bath envisaged that a microbial transformation system, capable of effecting the N-dealkylation of drug molecules, could be developed to overcome the difficulties experienced by medicinal chemists in preparing useful drug intermediates. The system may also be of value in drug metabolism studies.

The initial studies carried out by Sewell[18] were concerned with the development of a microbial process with the capacity to N-dealkylate a variety of pharmaceutically important compounds. He selected a series of 'model' compounds for the initial screening experiments with methylotrophic Pseudomonads. This enabled the N-alkyl function to be presented in several of the different chemical environments typically encountered in pharmaceuticals. The structures of the 'model' test substrates are shown in Figure 1.1. Trimethylamine (1) represented the simplest tertiary aliphatic amine. N-methylpiperidine (2) is a simple tertiary amine in a saturated cyclic structure. Codeine (3) is a naturally occurring alkaloid based on a phenanthrene ring structure which is common to the family of morphine-like analgesics. In addition to the N-methyl functions, it also possesses a phenolic methyl ether, thus offering sites for both O- and/or N-demethylation. Tropinone (4) represented the molecular nucleus typically found in the naturally occurring tropane family of alkaloids. Pentazocine (5) is a typical example of a 6,7-benzomorphan analgesic, and was included in the screening programme to determine the ability of the test microorganisms to cleave a bulky nitrogen substituent.

Screening experiments with methylotrophic Pseudomonads suggested that the N-dealkylating capability of these microorganisms was limited to simple aliphatic amines, such as trimethylamine, and the screening programme was extended to include strains of *Streptomyces* and fungi, mainly species of *Cunninghamella*.

Figure 1.1. Test substrates selected for screening experiments with methylotrophic Pseudomonads by Sewell [18]



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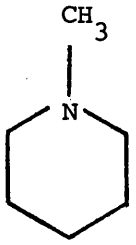

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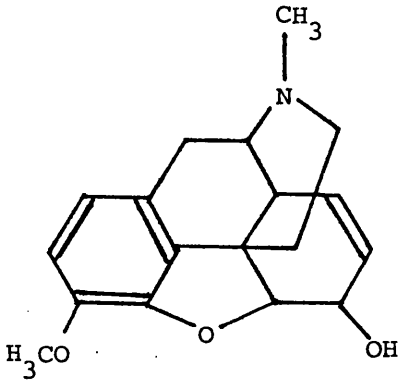

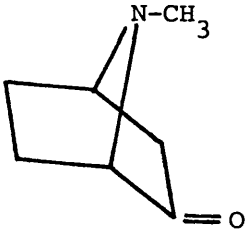

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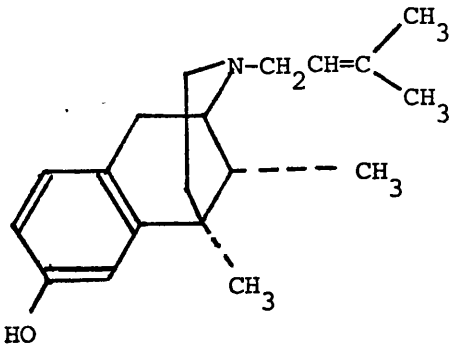


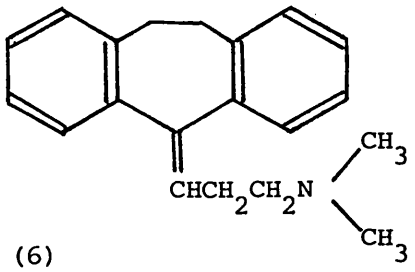
(5)

In these studies, he again selected 'model' compounds to be representative of the main classes of nitrogenous drug molecules of pharmaceutical interest (Figure 1.2).

Amitriptyline (6) a tricyclic antidepressant drug, with its dimethylamino group part of a propylidene side chain,may be N-demethylated to produce nortriptyline, and again to produce desmethylnortryptyline. Chlorpromazine (7), a central nervous system depressant, may be N-demethylated at the dimethyl-aminopropyl side chain to give the secondary amine (8) des-methylchlorpromazine, and again to produce the desdimethyl-derivative. In addition, the entire dimethylaminopropyl group may be cleaved from the phenothiazine nitrogen atom. Desmethyl-chlorpromazine (8) was screened separately as it presented a secondary amine system for transformation. Diazepam (9) is a member of the benzodiazepine group of minor tranquilizers, and was included because it is a cyclic amide, and not an amine. Metazocine (10) is a synthetic analgesic representative of the 6,7-benzomorphans. Codeine (3) was also screened to test the selectivity of microorganisms for O and/or N-demethylation. These microbial transformation experiments were conducted in submerged liquid culture, and demonstrated the ability of several of these microorganisms to N-demethylate the drug molecules.

A few of the *Streptomyces* species were able to transform one or two of the test compounds. *S. lincolnensis* N-demethylated codeine, metazocine, and amitriptyline to nortriptyline and also

Figure 1.2. Chemical structures of model substrate compounds selected for screening with Streptomycetes and fungi[18] by Sewell



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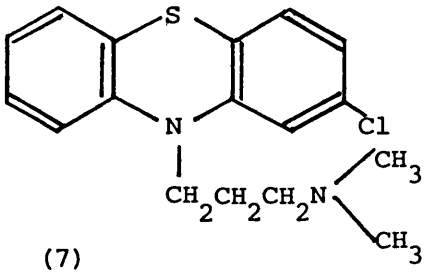

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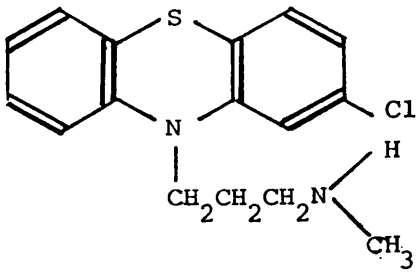

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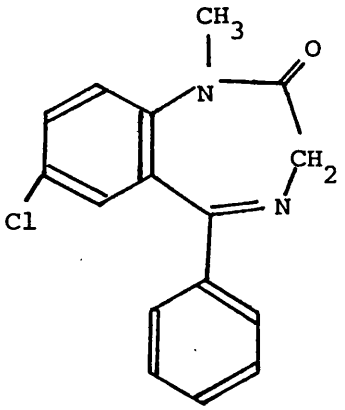

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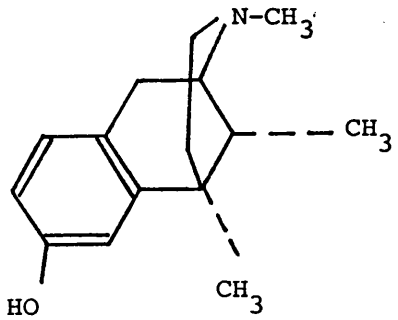


(10)

to desmethylnortriptyline, *S. rimosus* N-demethylated diazepam and amitriptyline, and *S. paucisporogenes* N-demethylated codeine only. However, the most promising microorganisms screened were the *Cunninghamella* since all test substrates, with the exception of pentazocine, were N-dealkylated by several species of this genus[18].

Continuing these studies, Sewell[18] developed defined and semi-defined growth media to facilitate the quantitative investigation of various growth medium parameters on N-demethylation. *C. echinulata IMI 199844*, was selected as the test organism since the strain had demonstrated the N-demethylation of a wide range of compounds with product yields estimated to be superior to those achieved by other active organisms. Codeine was selected as the substrate for these studies, and in order to quantify the transformation results, norcodeine, the transformation product, was estimated by GC assay, and the microbial growth by determination of dry cell weight. On the basis of both microbial growth and codeine transformation, Sewell[18] was able to ascertain the most suitable growth medium for further transformations. Transformation experiments conducted in culture media containing carbon sources which supported a rapid microbial growth rate , e.g. maltose, gave low conversions to norcodeine, whereas cultures containing a less utilised carbon source such as succinate produced higher transformation yields of norcodeine. A carbon source regulatory mechanism was suspected.

Casein hydrolysate, an organic nitrogen source, added to

the culture media, improved microbial growth and codeine transformation. Further investigations with the 13 individual amino-acids found in casein hydrolysate, omitting each one in turn from the culture media, showed that only the absence of leucine resulted in an increase in microbial growth and codeine transformation. This 'modified' amino acid mixture, containing the remaining 12 amino acids, was adopted in preference to casein hydrolysate, or inorganic nitrogen sources, such as ammonium sulphate and sodium nitrate, as it gave the highest yield of norcodeine.

The addition of a vitamin supplement to the growth medium produced no significant effect on transformation, and was subsequently omitted. However, the substrate concentration, in the medium was critical and concentrations above 1 mM reduced the transformation dramatically, probably due to substrate inhibition[18].

Further studies with *C. echinulata* disclosed that it exhibited typical pelleted fungal growth with cube root, rather than exponential kinetics. The growth curve, of dry cell weight against incubation time exhibited a short lag period, followed by a slow growth phase which continued up until the fourth day of incubation. Cell lysis, measured as the leakage of 260 nm absorbing substances from the cell, and the extracellular protein concentration, both increased until the seventh day of incubation and then levelled off. This suggested that codeine transformation was not dependent upon cell lysis as a means of enabling contact between the codeine

substrate and N-demethylating enzyme system. The extent of fungal growth was found to be limited by carbon source exhaustion which occurred between the fifth and sixth days of incubation. N-demethylation occurred only after the glucose carbon source had been depleted, providing further evidence for the presence of a carbon source regulatory mechanism. This was attributed to catabolic repression rather than catabolic inhibition, supported by subsequent experiments with resting cell suspensions. Cells harvested from cultures in which carbon source depletion was incomplete were unable to effect significant codeine N-demethylation when resuspended in basal-salts medium, but cells harvested from cultures in which glucose was exhausted demonstrated comparable codeine N-demethylation to that obtained with growing submerged cultures. These findings suggested an absence of preformed N-demethylase in cells harvested prior to glucose depletion in the culture medium.

Since *C. echinulata* IMI 199844 had demonstrated N-demethylase activity with a variety of different substrates in the screening programme, Sewell[18], proceeded to attempt to characterise the enzyme responsible for N-demethylation. To do this it was necessary to extract the N-demethylase from the fungal cells in order to study the enzyme, independent of enzyme production and cell permeability effects.

A cell-free extract was prepared from *C. echinulata* which retained a measurable degree of N-demethylase activity. The success of various enzyme extraction procedures was measured in

terms of the supernatent protein concentration after the removal of cell debris, and the determination of the formaldehyde produced by codeine N-demethylation with the cell-free extract.

Sewell[18] found the most effective extraction procedure was homogenisation, with subsequent treatment of the homogenate with Triton X100,a nonionic surfactant. The homogenisation process was observed to disrupt the mycelial pellets, and the Triton X100 was thought to solubilize the hydrophobic part of the enzyme associated with the cellular membrane fragments. Fungal monooxygenase enzymes which are frequently implicated in oxidative transformations have been shown previously to be membrane associated systems[19,20].

The N-demethylase activity of cell-free extracts was increased by the addition of Fe^{2+} to incubation mixtures, but surprisingly reduced by the addition of NADPH. This was thought to be due to an inhibitory effect of NADPH, also reported by Edelson and McMullen[21] for O-demethylation of p-nitroanisole by *E. coli*, but the reasons for this observation remain uncertain. The *C. echinulata* cell-free extract exhibited a broad temperature optimum of around 30^{o} - 32^{o} C, and a pH optimum of 7.5, for the N-demethylation of codeine. The active component of the cell-free extract behaved as a typical monooxygenase enzyme since codeine N-demethylase was inhibited by anoxic conditions, CO and SKF 525A. Furthermore, carbon monoxide and SKF 525A are classicial inhibitors of monooxygenases whether of mammalian[22,23] or microbial[24,25] origin, therefore Sewell suggested, although

he could not confirm, that the *C. echinulata* codeine N-demethylase maybe a typical cytochrome P-450-linked monooxygenase.

1.5 Processing and isolation of the transformation product

It is usually necessary to scale up a microbial transformation in order to produce usable quantities of the desired transformation product[26]. The scale of the fermentation employed will be dependent on the objectives of the project. Screening studies are usually conducted in small shake flasks containing 5 - 50 ml medium, whilst preparative scale studies may be conducted in large Erlenmeyer flasks , in multi-litre stirred laboratory fermenters or in large industrial fermentation equipment[27]. In addition to the biological and engineering problems associated with the scale of microbial transformations considerable difficulties may arise in the detection and analysis of the transformation product in relatively large volumes of medium. Often the substrate is expensive,or in short supply, or the transformation yield may be unavoidably low. In these cases it may be necessary not only to detect and assay the transformation product in the presence of the substrate and the growth medium components, but also to quantitatively separate the product from the substrate to facilitate recycling of the untransformed substrate. Separation and purification procedures can be expensive and time consuming especially on an industrial scale and the economic viability of a microbial transformation process will depend upon the careful selection of such procedures.

The choice of the most rapid and convenient separation procedure requires careful evaluation of many factors which include properties of the transformation product itself such as its stability, the scale of the operation, the time required, operation costs and material costs. A knowledge of the conditons under which the end product is stable is essential when selecting a separation procedure. Details of the effect of pH, temperature, solvent, and light on the compound are required[29]. Extraction procedures may involve drastic changes in pH, temperature or other laboratory stresses which could result in decomposition of the transformation product.

At the completion of the transformation the end product is almost always extra cellular and dissolved or suspended in the microbial growth/transformation medium.[28] In the case of fungal fermentation, the cellular material can usually be removed from the medium by filtration, but recovery of the end product requires a stepwise process of separations[29].

The relative affinity of a substance for two phases is the distribution coefficient, frequently expressed as the ratio of concentration in the two phases. The ratio of the distribution coefficients for two substances is the separation factor (α) for the substances in the system under consideration. If the separation factor value is very large, it is generally termed a 'group' separation, and if it is small it is termed a 'fractionating'separation[29].

The overall isolation of an end product from a fermentation mixture usually requires 'group' separation, followed by a 'fractionating' separation. Initially , the group separation results in enrichment of related substances from the mixture, including the end product, unconverted starting material and some constituents of the nutrient medium. This process should be as selective as possible to eliminate many unwanted compounds. The application of the fractionating process serves to isolate the end product in high purity and yield.

Substances encountered in transformation studies can usually be group separated based on their ionic or polar properties, often utilising a solvent extraction technique[28]. However, many solutes of biological or pharmaceutical interest are weak acids or bases and their extraction from aqueous environments such as transformation mixtures has proved difficult by conventional extraction procedures. Other classical group separation techniques such as freeze-drying and steam distillation have serious limitations in terms of recovery, capacity and possible loss of sample during processing, especially for temperature-sensitive compounds[30].

More efficient separation procedures have been developed which involve the adsorption of the product onto a solid surface Recoveries of 90 - 100% from aqueous samples have been reported for a wide range of organic molecules using a macro-reticular XAD-2 resin packed in glass tubes[31]. Other materials such as

XAD-4 resins, spherocarb and HPLC packing materials have also been used successfully for sample enrichment from biological media[32]. The aqueous sample containing the end product is injected onto the adsorption surface and the species becomes immobilised until the elution strength of the solvent mixture is increased. When the sample is eluted it is concentrated, prior to the application of the final fractionating step.

A fractionating separation method is required to produce a product of high purity and good yield. To achieve this a counter-current contact of the two phases is necessary, utilising a chromotographic method. Over the years, chromatography, in its various forms, has found wide application to the analysis of drugs in complex solutions because of its ability to isolate the compounds of interest. Gas liquid chromatography (G.L.C.) has been pre-eminent in the field, although it has always lacked the selectivity enjoyed by liquid chromatography with its wide choice of stationary and mobile phases.

1.6. High-performance Liquid Chromatography (H P L C.)

1.6.1 Advantages of HPLC in transformation studies

The advantages of High Performance Liquid Chromatography (H P L C), like gas chromatography (G C) lie in the combination of sensitivity, efficiency, reliability and speed of analysis of the technique . A major advantage of H P L C. over

G.C. is the versatility of the technique. As there are a wide range of stationary and mobile phases available, HPLC may often achieve separations that are impossible by G.C. In addition, many organic compounds are too unstable or insufficiently volatile to be handled by G.C. without prior modification or derivatisation. Although derivatisation is not required for separation in HPLC, sensitivity can be dramatically enhanced by the formation of strongly U.V. absorbing[33-34] or fluorescent derivatives[35-36] (either chemical or physicochemical e.g. ion pairing).

There is a wide choice of detectors available for use in HPLC The most widely used detector is the ultra-violet (U.V.) photometer[29], but others include electrochemical, fluorescence and refractive index detectors. Many of these detectors are selective so a complete separation need not necessarily be made on the column but a detector can be chosen that will monitor only the species of interest.

Finally, recovery of the sample in HPLC can be achieved more easily and quantitatively than in G.C., and this is possible on a much larger scale using preparative liquid chromatography. The lower temperatures that are used in HPLC complement the mild conditions used in microbial transformations when dealing with heat labile products. In addition, HPLC columns tend to have a long working life without regeneration. These particular advantages make HPLC a most suitable fractionating separation procedure to use in transformation studies.

1.6.2 Stationary phases

Liquid chromatography phase systems may be described as being either straight (or normal) phase or reversed phase, according to the nature of the mobile and stationary phases. In straight phase systems the stationary phase is more polar than the mobile phase, which typically is a mixture of organic solvents. In reversed phase, the stationary phase is non-polar and the mobile phase is polar, usually aqueous, in combination with an organic modifier.

In HPLC the stationary phase may be a pellicular[37] or porous solid such as silica[38], alumina[39] or carbon[40], a resin[41] or a liquid physically bound to an inert support. The development of chemically bonded phases constitute another type, and are now the most widely used[42]. The majority of modern bonded phases are prepared from silica by reacting the surface silanol groups with an organochlorosilane or alkoxysilane to give an Si-O-Si-R linkage which is hydrolytically stable. The R group is usually a straight chain hydrocarbon of known and uniform chain length (e.g. C8 or C18) or a hydrocarbon with a polar terminal group (e.g. R-CN, $R-NH_2$). Thus the bonded phase may range from polar to non polar depending on the type of terminal group.

Bonded phases may possess cross-linkages between the hydrocarbon chains, and these are described as being polymeric in form. More commonly, the bonded moiety is arranged at right

angles to the solid surface to achieve a 'brush-type' orientation. The Si-O-Si bond is generally stable over a pH range of 2 to 7.5. which can limit its usefulness. The polar hydroxyl groups remaining on the surface of the silica after bonding can be removed or 'capped' by reaction with a small chlorosilane such as trimethylchlorosilane. Both capped and uncapped bonded phases are available commercially in a range of products.

1.6.3 Retention mechanisms

a) Liquid solid adsorption chromatography:

Polar inorganic materials such as silica or alumina are most often used, and the mobile phase is predominantly an organic solvent mixture. The surface of silica consists primarily of slightly acidic silanol groups which may be associated with water molecules via hydrogen bonding. Silica is heated to 150^{o}C to drive off the associated water and increase the surface activity. Retention and selectivity are the result of adsorption of the polar or polarizable functional groups of the solute molecules directly onto the surface of the packing. The adsorption interaction is the displacement of mobile phase molecules from the adsorbent surface by the solute in a competitive reaction. In the absence of steric effects, solute retention is proportional to the relative number and type of polar functional groups in the solute.

This mode of chromatography has an important role in thin layer chromatography, preparative scale column chromatography

and straight phase High Performance Liquid-Solid Chromatography (HPLSC) in the separation of moderately polar solutes.

(b) Partition (Liquid-Liquid Chromatography):

Retention of the solute is due to the partitioning between the mobile phase, and the organic phase physically coated onto the surface of the particle. The stationary phase is usually polar, and is immiscible with the mobile phase. These phases are rarely used today having been superseded by chemically bonded phases.

(c) Bonded phase chromatography:

Bonded phases with non-polar functional groups (C_1-C_{18}) are the most widely used materials in HPLC, especially hydrocarbons C_8 and C_{18} or octadecylsilane (ODS). These are available as a range of products that vary in their percentage carbon loading, the extent of silanol capping, the surface area, and the particle size and shape of silica to which it is bonded. The mobile phase is usually aqueous and buffered at a pH that enhances selectivity. It may contain an organic modifier,such as methanol or acetonitrile to adjust the retention. It has been shown that provided the amount of organic modifier is enough to wet the surface of the bonded phase, then a monolayer of organic modifier is adsorbed at the hydrocarbon surface[43]. This layer is not displaced by solute and so must take part in the retention mechanism.

However, the retention mechanism is complex and not fully understood. It has been proposed[44] that solutes having some

hydrophobicity are squeezed out of the aqueous mobile phase and into the hydrocarbon brush because solute interactions with water are weaker than interactions between water molecules. This is called the Hydrophobic Effect. Increases in carbon loading and chain length enhance the hydrophobic surface area and hence retention. Interactions between polar functional groups on the solute and water will reduce retention, and explains why pH control is important when dealing with ionizable solutes.

(d) Ion-Pair Chromatography

This is a special form of Liquid-Liquid Chromatography used for the separation of ionic or ionizable compounds. In its most usual form it is used in the reversed phase mode, in which the aqueous mobile phase containing organic modifier, buffer and pairing ion is passed through a hydrocarbon bonded stationary phase. The mechanism of retention is subject to debate[48-51], although two mechanisms have been proposed, the partition mode and the ion-exchange mode. In the partition mode, the ionizable solute has insufficient lipophilic character to be retained, and forms an ion-pair with a suitable counter-ion (or ion-pair agent) added to the mobile phase. The ion-pair has increased lipophilic character and increased affinity for the stationary phase. In the ion-exchange mode the polar counter-ion is assumed to be absorbed by the hydrocarbon stationary phase to create an ion-exchange site onto which the polar sample molecule is adsorbed much as in ion-exchange chromatography.

In ion-pair chromatography the control of pH, the size and nature of the counter-ion, and to a lesser extent, the nature of the stationary and mobile phases all have an effect on the degree of selectivity. In the pH range 2 - 8 strong acids and bases are completely ionized, weak acids and bases will be ionized depending on the pH chosen. If the equilibrium

$$\text{non ionic} \rightleftharpoons \text{ionic}$$

lies to the left they elute as non-ionic components on a non-polar stationary phase. If the equilibrium lies to the right they can interact with the ion-pair agent as for strong acids or bases.

1.6.4 Definitions

The following expressions have been employed in order to assess various chromatographic parameters used in this thesis.

(a) Retention:

The term used to describe solute retention in column and gas chromatography is the column capacity ratio, κ. The retention and therefore the separation of components in a mixture will depend on the distribution of each component between the two chromatographic phases.

$$\kappa = \frac{Cs.Vs}{Cm.Vm} = D \frac{Vs}{Vm} \qquad \ldots 1.1$$

where C and V are the solute concentrations in, and the volumes of, the mobile phase, m, and the stationary phase, s, respectively.

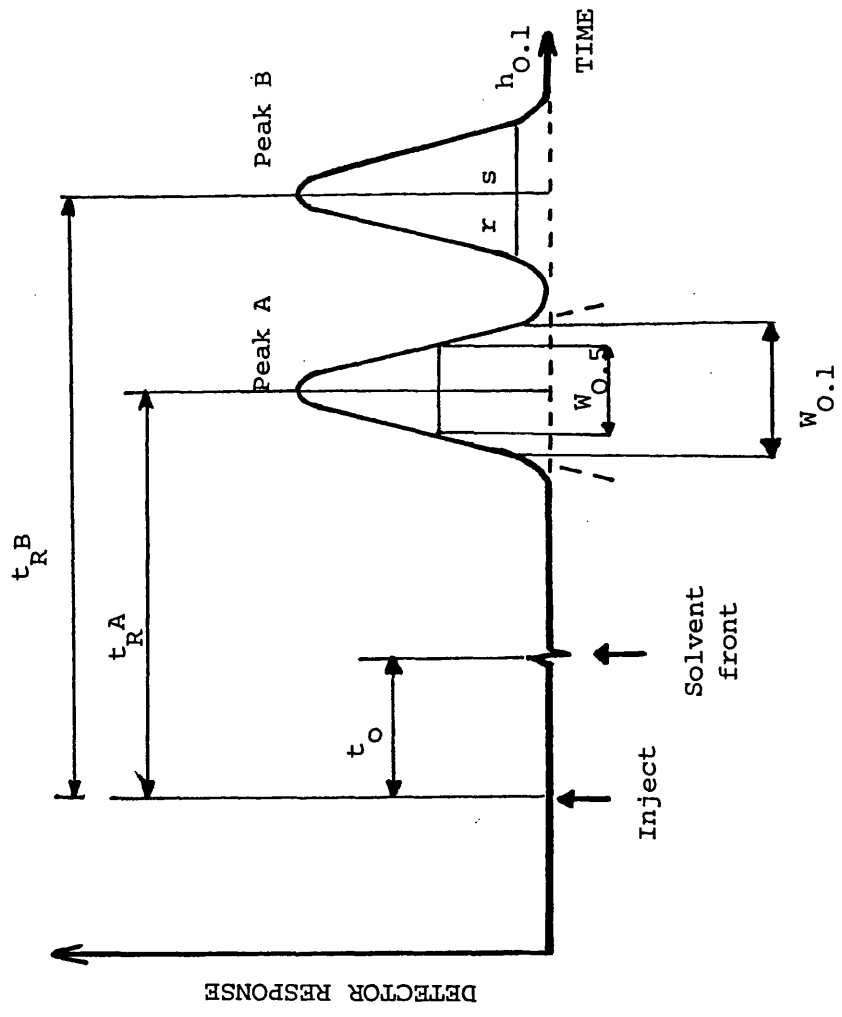


Figure 1.3. Peak Measurements

D is the distribution ratio of the solute between the two phases. In practice the capacity ratio may be determined from

$$\kappa = \frac{(t_R - t_o)}{t_o} \qquad \ldots 1.2$$

where t_R and t_o are the elution times or chart distances of the retained or unretained peaks respectively (Fig. 1.3).

(b) Band broadening:

As a solute band passes through the column, the width of the band increases as the solute is diluted by the mobile phase. Band width, W, is commonly expressed in terms of the theoretical plate number, N, of the column and is a useful measure of column efficiency, i.e. the relative ability of a given column to provide narrow bands and improved separations. The number of theoretical plates N of a chromatographic system may be defined from a single chromatographic band(Fig. 1.3)

$$N = 16 \left(\frac{t_R}{W_{0.1}}\right)^2 \quad \text{or} \quad 5.54 \left(\frac{t_R}{W_{0.5}}\right)^2 \qquad \ldots 1.3$$

where t_R, $W_{0.1}$ and $W_{0.5}$ are the retention time, width at 0.1 of the peak height ($h_{0.1}$) and width at half the height ($h_{0.5}$) of the peak, respectively. The peak width measurement $W_{0.1}$ takes into account peak tailing at the lower parts of the elution curves, and gives a more realistic measurement of column efficiency and resolution, especially in the evaluation of preparative chromatographic systems[45].

In comparing column efficiencies a more useful parameter is the height equivalent to a theoretical plate (HETP) or plate value (H)

$$H = \frac{L}{N} \qquad \ldots 1.4$$

where L is the length of the column and H measures the efficiency of the column per unit length.

The reduced plate height, h, is a measure of column packing performance taking into account the particle size, dp,

$$h = \frac{H}{dp} \qquad \ldots 1.5$$

where dp is the diameter of the stationary phase particles.

(c) Velocity:

The flow rate (ml/min) is a measure of the volume flow of mobile phase through the column. Linear velocity (u) is a measurement of flow rate independent of the cross-sectional area of the column

$$u = \frac{L}{t_o} \qquad \ldots 1.6$$

and the reduced velocity, v is a term independent of particle size and mobile phase characteristics, e.g. viscosity, given by

$$v = u \frac{dp}{Dm} \qquad \ldots 1.7$$

where Dm is the diffusion coefficient of the mobile phase.

Experimental values of reduced plate height h obtained from Equation 1.5 plotted against reduced velocity v of mobile phase for a given solute and set of conditions, produce a typical Knox curve.This can be used to determine the most efficient flow rate for a particular sample (Fig. 1.4).

(d) Resolution and Selectivity:

The degree of resolution R_s of two adjacent solute bands is defined by the empirical relationship

$$R_s = \frac{t_{RB} - t_{RA}}{\frac{1}{2}(W_B + W_A)} \qquad \ldots 1.8$$

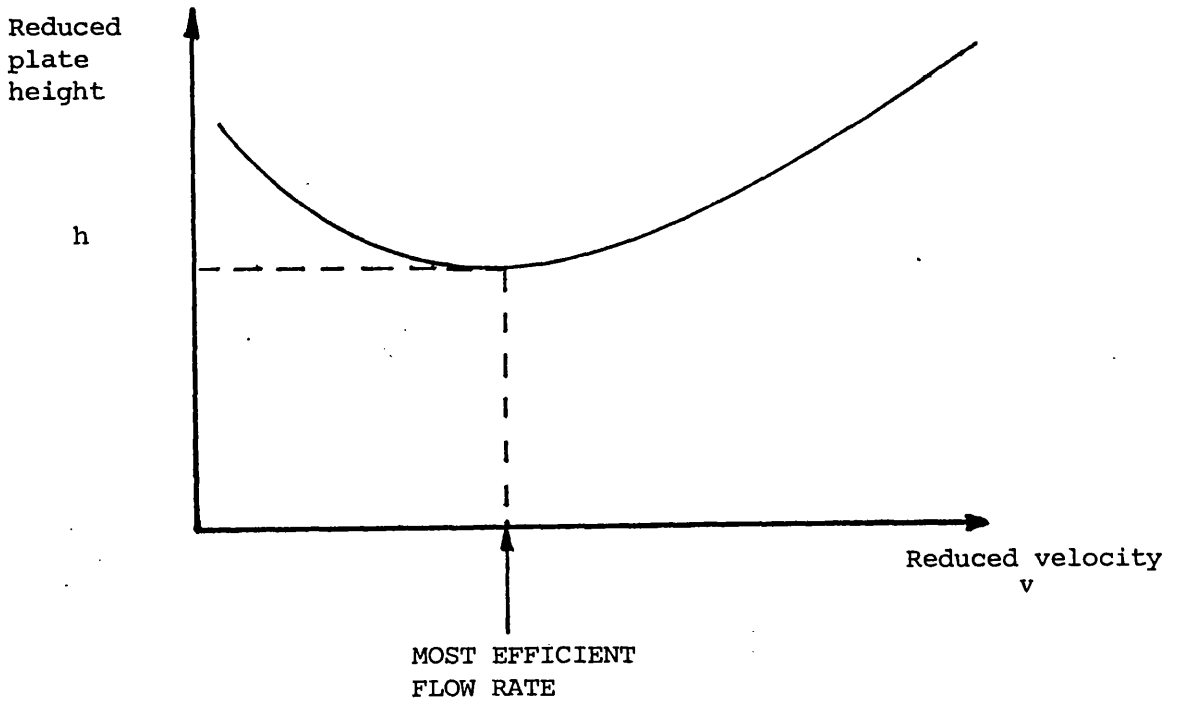
where t_R is the elution time or chart distance of solutes A and B and W is the width at the base of their peaks (Fig. 1.3). In theory an R_s value of 1 would give complete separation at the baseline, but in practice, because the peaks are Gaussian in shape, a R_s value of 1.2 is usually required for a base line separation.

Purnell[47] has shown that for column chromatography the resolution (R_s) can be related to the average capacity ratio (κ), the relative retention (α) and the number of theoretical plates (N) by the relationship:

$$R_s = \frac{1}{4} \underset{(i)}{\frac{\alpha - 1}{\alpha}} \cdot \underset{(ii)}{\sqrt{N}} \cdot \underset{(iii)}{\frac{\kappa}{1+\kappa}} \qquad \ldots 1.9$$

where the relative retention value α compares the degree of retentivity of one peak with another and is given by:

Figure 1.4. Plot of reduced height (h) versus reduced velocity (v) (or Knox curve)[46] used to estimate the most efficient flow rate to use in a liquid chromatography separation



$$\alpha = \frac{K_B}{K_A} \quad \text{for peaks A and B} \qquad \ldots 1.10$$

Each term (i) to (iii) in equation 1.9 can have an independent effect on resolution. The column selectivity (term (i)) is varied by changing the composition of the mobile and/or stationary phases, and to a lesser extent, the temperature. An increase in column efficiency N (term (ii)) will increase the resolution. This is achieved by using a longer column, reducing the solvent velocity or employing a different column packing material. Finally, relative retention (term (iii)) is varied by changing the mobile phase composition.

(e) Peak shape:

The symmetry factor at 10% of the peak height ($A_{10\%}$) can be described as

$$A_{10\%} = \frac{2r}{r + s} \qquad \ldots 1.11$$

where r and s are measurements at 10% of the peak height illustrated in Fig. 1.3. Ideally a value of $A_{10\%}$ of 1 is required. The effect of sample loading on peak shape and symmetry are reported in Section 2 of this thesis.

(f) Functional Group Contribution Value:

Functional group contribution towards retention may be defined as

$$\tau = \log \alpha = \log \frac{K_2}{K_1} \qquad \ldots 1.12$$

where K are the capacity ratios of solutes 1 and 2 which differ by a functional group. The reference solute 1 is taken as the parent compound. Values of τ for a series of 1,4-benzodiazepines are reported and discussed in Section 7.5.

(g) Porosity:

The total porosity of a column (ε) is the sum of the interparticle porosity (ε_I) and the porosity of the individual particles (ε_p) where:-

$$\varepsilon_I = \frac{\text{volume of spaces between the particles}}{\text{volume of the column tube}} \quad \ldots 1.13$$

and $$\varepsilon_p = \frac{\text{volume of pores}}{\text{total volume of the particle}} \quad \ldots 1.14$$

so $$\varepsilon = \varepsilon_I + \varepsilon_p \quad \ldots 1.15$$

In practice the total porosity (ε) can be determined from

$$\varepsilon = \frac{Vm}{(\frac{\pi}{4})\, d_c^{\,2} u} = \frac{F_c \times t_o}{19.625 \times L} \quad \ldots 1.16$$

where F_c is the flow rate in ml/sec, t_o is the elution time of an unretained peak (seconds) and L is the column length in metres. The total porosity is estimated for all newly packed columns.

1.7 Nuclear Magnetic Resonance (N.M.R.)

1.7.1 General principles of N.M.R.

The basic principles of Nuclear Magnetic Resonance (NMR) are reviewed in several spectroscopy reference books[52,53], hence, the following is a brief summary of the necessary concepts.

Many atomic nuclei possess intrinsic angular momentum, a property characterized by the spin quantum number (I). For such nuclei there are 2I + 1 allowed spin states. Those nuclei with I = ½ (^{1}H, ^{13}C, ^{15}N, ^{31}P) fall into the most common NMR category where only two states are possible. In the presence of an applied magnetic field these spin states correspond to different energy levels and it is the transitions between these levels that are detected by NMR spectroscopy. In the NMR experiment the net absorption of electromagnetic radiation by the nuclear spins is detected as a resonance frequency between the two spin states. The frequencies at which signals are observed depend on the energy differences between spin states, which in turn are dependent on the magnetogyric ratio (γ) of the nucleus under observation, the strength of the applied field, and the electronic environment of the resonant nucleus.

Excited nuclei return to their ground spin population in two ways. By transfer of energy to neighbouring nuclei in the molecular framework or lattice (spin-lattice relaxation), with an associated time constant (T_1), the spin-lattice relaxation

time. Secondly, by mutual exchange of spins between excited nuclei and neighbouring nuclei (spin-spin relaxation) with an associated spin-spin relaxation time (T_2), which affects the natural line width of an NMR absorption peak.

The field experienced by a particular nucleus in a molecule differs from the applied field by a small amount depending on the screening (deshielding) effect of surrounding electrons. The extent of deshielding, the chemical shift, is expressed relative to a standard in parts per million (ppm) and is thus independent of the applied field. ^{13}C chemical shifts are normally measured relative to tetramethylsilane (TMS, 0 ppm) and usually fall within the range 0 - 210 ppm.

NMR spectroscopy is a relatively insensitive spectroscopic technique compared with other spectroscopic techniques (U.V., I.R.)[52]. This is due to the small energy difference between spin states resulting in only a slight population imbalance in favour of the ground state, and because the signal is proportional to the population difference, a relatively small proportion of nuclei are accessible to the environment. Furthermore, as excited states are relatively long lived (in the order of 10^{-2} - 10^{8} sec. for ^{13}C), the application of excessive power leads to equalization of spin populations (saturation) with no net emission of energy.

There are also significant differences in sensitivity existing between nuclei. Hence, a ^{13}C nucleus gives rise to only

1/64th of the signal that a proton would yield. In addition, isotopes vary in their natural abundance. For example, ^{13}P and ^{1}H are present in nature in high isotopic abundance, whilst ^{13}C occurs as only 1.1% of total carbon.

1.7.2 Techniques for recording NMR spectra

To overcome the sensitivity problem it is possible to record several spectra from a sample and add them together. The signal-to-noise ratio (S/N) will improve by the square root of the number of spectra accumulated. Using digital computers to store and add the spectra it is possible to accumulate numerous spectra (scans) in this manner. Using a conventional continuous wave (C.W.) spectrometer, radiation at a single frequency is slowly swept across the magnetic field spectral width. The main disadvantage of this technique is the time taken to record a continuous scan from low field to high field (100 - 500 seconds). Consequently the time taken to obtain a spectrum can be incredibly long,sometimes prohibiting its use.

The advent of routine Fourier Transform spectrometers, equipped with a computer, has given a solution to the problem. In the pulse technique all the frequencies in a spectrum are irradiated simultaneously at constant magnetic field by employing a strong radio frequency pulse of 5 - 50 μsec. duration over a broad band width. When the pulse is switched off the nuclei undergo relaxation processes and re-emit the

energies simultaneously in a complex interacting pattern, which decays rapidly and is called the free induction decay (F.I.D.). This output is digitalised in a computer, and each individual frequency is filtered out from the complex pattern. Each of these frequencies in plotted on a linear frequency scale, which corresponds to the conventional nmr spectrum. The FID is a time-based process, a measurement of intensity as a function of time. Transformation to the frequency domain signal used in C.W. measurements can be carried out mathematically by a process known as Fourier Transformation (F.T.). This is achieved in modern spectrometers by the same computer which is used to accumulate and store the data. After excitation by one pulse, the nuclei cannot be re-excited until T_1 (the spin-lattice relaxation time) has elapsed. The time for this is usually short,approximately 2s. allowing for the pulse itself, acquisition time and pulse delay, and many pulses may be made over a relatively short period of time to produce a final spectrum with an acceptable signal-to-noise ratio.

1.7.3 Proton noise decoupling (COM)

The low natural abundance of carbon-13 may produce a sensitivity prcblem in carbon-13 magnetic resonance spectroscopy, but it also has one advantage. It is unlikely that two carbon-13 nuclei will be in adjacent chemical positions in the same molecular structure, so that the possibility of finding two coupled carbon-13 nuclei is negligible. The absence of carbon-

carbon couplings simplifies the spectra greatly.

However, carbon-proton (^{13}C-^{1}H) coupling does occur, and this can produce extensive multiplets which may overlap in the spectra. As a result it may be difficult to assign the peaks corresponding to different carbon-13 nuclei. In addition, the sensitivity is seriously reduced.

To overcome this problem, a proton noise decoupling technique is applied to simplify the spectrum. This procedure depends upon strongly irradiating all protons in the sample with a second intense band of radio frequency, so that their spins are effectively decoupled. The carbon-13 nmr spectra are then observed as single sharp lines rather than as complex multiplets. Since interaction with neighbouring nuclei (usually protons) is a major pathway for spin-lattice relaxation of ^{13}C nuclei, irradiation at proton resonance frequencies will increase the population differences between spin states and lead to an enhancement of ^{13}C signal strength. This phenomenon is known as the Nuclear Overhauser Effect (NOE). The signals of ^{13}C nuclei directly bonded to protons give a NOE of approximately 3x, in addition to enhancement brought about by collapse of spin multiplets.

1.7.4 Off-resonance decoupling (OFR)

Proton noise decoupling simplifies the nmr spectra, but all the coupling information is lost, so that fully decoupled

spectra may be difficult to assign. One way of overcoming this problem is by employing the technique of off-resonance decoupling (OFR). A partially decoupled spectrum is obtained by setting the proton decoupling frequency between 1000 - 2000 Hz above TMS (i.e. 1000 - 2000 Hz outside the proton region) and turning the noise modulation off. Under these conditions all the ^{13}C - ^{1}H couplings (H attached directly to carbon) and these one-bond couplings will be reduced to about 30 - 50 Hz in magnitude. Hence, primary carbons will appear as quartets, secondary carbons as triplets, tertiary carbons as doublets and quaternary carbons as singlets.

1.8 Applications of ^{13}C N.M.R. to biosynthetic and metabolic studies

NMR spectroscopy offers several advantages when applied to biosynthetic problems as it is probably the only technique which can separately detect the presence of isotopes of all biosynthetically useful elements (^{1}H, ^{2}H, ^{13}C, ^{15}N, ^{17}O, ^{31}P etc.)[56]. Like other spectroscopic techniques NMR is non destructive, so samples can be recovered and utilised for other analytical techniques[57]. Furthermore, it is capable of directly determining the locations and concentrations of isotopic labels in a metabolite. Spin coupling effects can provide direct evidence for the incorporation of intact biogenic units, and for biosynthetic processes involving bond formation and cleavage. Such information can only

be deduced indirectly by other methods[56]. The use of ^{13}C spin-lattice relaxation data (T_1) can provide a considerable aid to the assignment of carbons, for example, in molecules containing large numbers of carbon atoms, where serious overlapping of the multiplet structures limits the use of off-resonance decoupling.[54]

The availability of materials enriched with istopes, ^{13}C in particular, has simplified biosynthetic studies of microbial metabolites[58]. It is possible to follow the incorporation of ^{13}C labelled precursors into molecules by means of ^{1}H and ^{13}C NMR, and in many cases, spectral analysis of the labelled product permits detection and identification of the incorporation sites, without need for chemical degradation[57]. Biosynthetic information can be obtained from the assignment of ^{13}C resonances. Selective enrichment may simplify assignments as chemical shifts and coupling patterns (e.g. in off-resonance decoupled spectra) may be more easily identified following enrichment. Ambiguities due to partial overlap can often be resolved. For example, biosynthetic enrichment has been used to assign resonances in the complex spectrum of gramicidin[59], and chemical introduction of ^{13}C labels was used to distinguish the resonances of the four meso carbons in protoporphyrin IX[60].

1.8.1 Labelling techniques

Labelling with stable isotopes, for example ^{13}C employs

similar procedures to radioisotope labelling.However, it is normally necessary to obtain a higher degree of ^{13}C enrichment, because ^{13}C measurements are far less sensitive than radioactive ^{14}C labelling techniques, and also because ^{13}C enrichments are measured against a 1.1% ^{13}C natural abundance background.

The degree of enrichment of ^{13}C into the precursor over the natural abundance of 1.1% depends on the amount of ^{13}C-labelled product required for identification. Normally, the level of enrichment must be 0.5 - 2% ^{13}C above the natural abundance to be detectable. High enrichment levels (e.g. 90% ^{13}C) allow a lower yield of metabolite to be detected. The simplest way of incorporating ^{13}C labelled atoms is to employ a precursor containing a single specifically enriched carbon atom. For example, using ^{13}C enriched acetic acid as the precursor it is possible to employ molecules selectively labelled at either the 1 or 2 position (* denotes the site of enrichment)

$*CH_3 - COOH$	$CH_3 - *COOH$
2 - ^{13}C acetic acid	1 - ^{13}C acetic acid

The creditability of the ^{13}C method can be illustrated by a study on the biosynthesis of Cytochalasin B, a member of a group of compounds displaying cytostatic activity[56]. Radiotracer studies showed that ^{14}C acetic acid is incorporated into Cytochalasin B, but the distribution of label from acetate was only partially revealed by degradation steps. In contrast, all sites enriched by 2 - ^{13}C acetic acid were readily detected by ^{13}C NMR and

confirmed the stages of the biosynthetic pathway.

Most applications of ^{13}C NMR have been confined to metabolites produced by microorganisms[56], because of good precursor incorporation and high yield of product, but any system which produces a sufficient yield of reasonably enriched material can be used[56].

It is also possible to employ precursors containing two (or more) enriched positions, such as doubly labelled acetic acid ($*CH_3*COOH$, 1,2 - ^{13}C acetic acid)[56]. If the two enriched ^{13}C atoms are directly bonded to one another, then ^{13}C - ^{13}C coupling will be observed between the two labelled positions, and the fate of the enriched substrate can be determined by the absence or presence of this coupling in the enriched metabolite. For example, if coupling is preserved in the ^{13}C spectrum of the labelled product it is good evidence that at least part of the precursor is incorporated intact. Loss of $^{1}J_{C-C}$ coupling accompanied by enrichment is proof of bond scission, and changes in the type of coupling (e.g. $^{1}J_{C-C} \rightarrow {}^{3}J_{C-C}$) may indicate a rearrangement.

Doubly labelled precursors in NMR have been employed in the study of rearrangements[61], as an aid in structure elucidation[62], and to distinguish biogenic alternatives[63].

1.8.2 The scope and potential of NMR in metabolic studies

The first applications of NMR to biosynthetic problems[64,65] in the late sixties traced the fate of ^{13}C enriched precursors by the increases in ^{13}C - ^{1}H intensities in the ^{1}H NMR spectrum. However this method was seriously limited by the inability to provide information for carbons not directly bonded to hydrogen, and was soon superseded by continuous wave (CW) ^{13}C NMR spectroscopy[66,67]. This permitted every enriched site to be identified directly from differences in the intensities of corresponding resonances in the ^{13}C NMR spectra of naturally occurring and labelled metabolite. A major advance was provided by the increase in sensitivity resulting from the development of pulse Fourier transform (PFT). ^{13}C NMR. This powerful technique has allowed many new areas of biosynthetic and metabolic interest to be explored, such as the study of metabolism in living cells, achieved as early as 1972[68].

However, the rate of progress of NMR metabolic studies *in vivo* has been hindered partly due to problems associated with obtaining well-resolved ^{13}C NMR spectra in salt solutions and biological fluids. Differences in magnetic susceptibilities within the biological sample tend to distort the magnetic field, and result in losses in sensitivity and resolution. Association of ^{13}C nuclei with certain paramagnetic ions can also result in greatly broadened signals[55]. To produce a well defined NMR spectrum by the PFT technique, the pulse-FID collection sequence is generally continued until adequate signal-to-noise (S/N) is achieved, but

a limiting factor for data accumulation is the relaxation rate of the species under examination. A second pulse given prior to re-establishment of equilibrium magnetization (T_1) will result in a reduction of signal intensity (saturation). Hence the longer the T_1 of the nucleus, the longer the time interval required between pulses. Since T_1 values for all carbon atoms of a molecule may be different, in practice a compromise has to be made between the pulse interval and the S/N ratio achieved in the shortest time.

Finally, in addition to the instrumental problems, there have been difficulties associated with peak assignment of an unknown and possibly transient species, that, in the case of a singly enriched substrate may give rise to only one resonance whose chemical shift does not correspond with the anticipated value.

Despite these problems, ^{13}C NMR studies have been successfully performed using a variety of living biological systems including tissues (e.g. mammalian nerves[69], arterial tissues[70]), whole organ preparations (e.g. perfused mouse liver[71]) and whole organisms (e.g. tobacco budworm[55]). ^{13}C NMR techniques have also been employed as a tool for the study of microbial metabolism employing both intact cells in suspension[72] and cell-free extracts[56,58]. This has been achieved by following the fate of a particular carbon atom of an enriched substrate molecule and analysing the time-flux spectra. Apart from precise measurements of the rates of metabolic processes, direct examination of ^{13}C

distribution in intracellular metabolic products enables the degree of randomization as a function of time to be accurately assessed.

In their now classic experiment Shulman *et al.*[73] followed the course of 1 - ^{13}C glucose metabolism in packed *E. coli* (MRE 600) cells. Under rapid pulsing conditions at high field (90.5 MHz; 0.3 sec. 60° pulses), they were able to obtain well-resolved spectra of anaerobic cells with one-minute accumulations. Signals for the C-3 of lactate and C-2 of succinate were observed 2 minutes after the addition of labelled glucose, while that for C-1 of fructose diphosphate (FDP) was observed after 13 minutes. The intensity of the FDP C-1 signal was observed to decrease concomitantly with the increase of lactate C-3, alanine C-3, valine C-3, valine C-4, 4', ethanol, and acetate C-2 signals. Subsequent oxygenation of the cell suspension after all the glucose had been consumed led to slow consumption of lactate with accumulation of glutamate labelled in the C-2, 3 and 4 positions[73].

The glycolytic process in red blood cells has also been the subject of some study. Styles *et al.*[74] monitored the events following 1 - ^{13}C glucose addition to human erythrocytes by simultaneously acquiring ^{13}C and ^{31}P spectra using a specially modified probe. Thus the accumulation of 3 - ^{13}C lactate and 3 - ^{13}C -2,3-diphosphoglycerate (DPG) was observed at the same time as the utilization of ATP, ADP, and the production

of inorganic phosphate.

Recently Cohen *et al.*[71] demonstrated that ^{13}C NMR could be applied to the study of whole organs. They determined the effect of ethanol on alanine metabolism in perfused mouse liver by following the fate of enriched carbons from 3 - ^{13}C alanine in the presence and absence of unlabelled 2 - ^{13}C ethanol. From the spectra accumulated over 30 minutes they demonstrated that in the presence of ethanol, label from 3 - ^{13}C alanine entered the tricarboxylic acid cycle exclusively through pyruvate carboxylation, and ethanol was the sole precursor of acetyl-CoA. However, in the absence of ethanol, alanine was observed to flow through both the tricarboxylic acid cycle and the pentose cycle.

While an immense amount of information has been abstracted from time dependent, high resolution spectra of cells and intact organs, significant results have also been achieved at lower resolution on whole organisms. For example, Scott and Baxter[55] performed a time course experiment on a single larva of the tobacco budworm, *Heliothus virescens* (F.), after the administration of 20 μM 1 - ^{13}C glucose. Spectra (21.5 MHz) taken at 0.5, 3 and 12 hours after injection of label clearly showed the flux of glucose into α , α'-trehalose, the major blood sugar of the insect.

Finally, ^{13}C NMR spectroscopy has been employed to detect the presence of transient or unstable intermediates in metabolic

studies. Ugurbil *et al.*[73] used ^{13}C-enriched glucose and a high-frequency ^{13}C NMR spectrometer to monitor metabolism in aerobic and anaerobic *E. coli* suspensions. The time-course of glucose metabolism was followed in well-resolved spectra obtained with 1-minute accumulations, enabling the intermediates as well as end products to be identified. These NMR experiments determined *in vivo* the extent of labelling among intermediates that may have rapid turnover rates, and thus avoided the uncertainties encountered in extracting such metabolites.

CHAPTER TWO

THE DEVELOPMENT AND APPLICATIONS OF HPLC TO THE STUDY OF MICROBIAL TRANSFORMATIONS.

CHAPTER 2. THE DEVELOPMENT AND APPLICATIONS OF HPLC TO THE STUDY OF MICROBIAL TRANSFORMATIONS

2.1 Introduction

Drug transformation studies often involve relatively small changes in substrate or endproduct concentration, especially in the early stages of transformation development. To monitor these changes accurate sensitive assay procedures were required, which were reproducible, and without interference from any of the test mixture components. Furthermore, the analysis time was required to be as short as possible.

A G.L.C. assay system was employed by Sewell[18] to study the N-demethylation of codeine by *C. echinulata*, and quantitatively estimate the norcodeine transformation product. However, there were several disadvantages associated with this procedure when used to assay drugs in transformation mixtures. It was necessary to extract the codeine and norcodeine from the transformation media, and also to derivatise the sample, prior to GLC analysis. This extended the analysis time, and the accuracy and reproducibility of the technique relied on the complete extraction and derivatisation of the sample each time. In addition, the GLC assay was found to be unsuitable for the analysis of norcodeine in cell-free extract studies. This was due to interference, attributed to Triton X-100 and its breakdown products, prohibiting quantitative determination of the transformation and also confusing the identification of the transformation product.[18]

Clearly, if further progress was to be achieved with these studies an alternative quantitative assay procedure was required. The potential benefits offered by HPLC, described in Section 1.6.1 of this thesis, could be utilised to overcome many of the inherent disadvantages associated with Sewell's GLC procedure.

The transformation of codeine by *C. echinulata* has been scaled up from shake flasks to a laboratory fermenter with a 7 litre volume capacity[18]. However, a suitable method had yet to be developed to efficiently recover and isolate the transformed drugs from the liquor. A further advantage of H P L C is that the sample can be recovered more easily than in GLC, and this is possible on a much larger scale using preparative H P L C This fractionating technique could be utilised for the final stages of purification of the transformation product from these larger volumes of transformation media.

For purposes of clarity this chapter has been divided into two sections. In the first section analytical HPLC methods developed for the quantitative determination of norcodeine and codeine in transformation media and cell-free extract mixtures are described. The second section describes the development of two complete systems to recover and isolate codeine and norcodeine from 7 litre batches of transformation media incorporating either straight phase or reversed phase preparative HPLC. Various column packing materials were also investigated for their ability to extract codeine and norcodeine to isolate these drugs prior to the application of the final preparative HPLC step.

Part A. Analytical HPLC Methods

2.2 Materials

2.2.1 Test compounds

Codeine phosphate; Macfarlane Smith Ltd., Edinburgh.

Codeine base; This was prepared from codeine phosphate by basifying (5 N NaOH) and extracting into 1,2-dichloroethane. The organic layer was dried (anhydrous $MgSO_4$) and evaporated to yield codeine base.

Norcodeine base; This was prepared by adapting the method used by Montzka *et al.*[75] to N-demethylate morphine. A solution of 2,2,2-trichloroethylchloroformate (15.0 ml) in dry toluene (30 ml) was added dropwise over 2 hours to a mixture of codeine base (5.7 g), very dry Na_2CO_3 (5.0 g) and dry toluene (75 ml) contained in a 250 ml. 3-necked flask. The mixture was refluxed for 48 hours with vigorous stirring, and then cooled to room temperature. Diethyl ether (100 ml) was added, and the whole was washed with 2 N HCl (2 x 100 ml) and water (100 ml). The organic layer was separated and evaporated to a yellow oil which was dissolved in glacial acetic acid (250 ml) and refluxed with zinc dust (18.0 g) for 1 hour. The mixture was stirred at room temperature for a further 4 hours and was then poured onto ice, basified (5 N NaOH), and extracted into 1,2-dichloroethane. The organic layer was dried (anhydrous $MgSO_4$) and evaporated to give an off-white solid (3.8 g). This was dissolved in the minimum quantity of hot acetone, filtered and allowed to cool to room temperature to yield a crystalline product (2.50 g).

Analytical: Melting point 184°C (lit[76] 185°C).

^{1}H NMR. H^{δ} (100 MHz; $CDCl_3$; Norcodeine base); 1.86 (2H, m, C_{15}-H); 2.7 (5H, m. C_{16}-H, C_{10}-H, C_{14}-H); 3.22 (2H, s, OH, NH, exchanges with D_2O); 3.63 (1H, m. C_9-H); 3.8 (3H, s, O-Me); 4.18 (1H, m. C_6-H); 4.88 (1H, d, J = 7.0 Hz, C_5-H); 5.29 (1H, q, C_7-H); 5.72 (1H, d, J = 10.5 Hz, C_8-H); 6.59 (2H, d.d, C_1-H, C_2-H).

Norcodeine HCl: This was prepared from norcodeine base, by precipitating it from a dry solution in 1,2-dichloroethane by the addition of ethereal HCl.

Analytical: m.pt. 308°C (lit[76] 309°C)

2.2.2 Internal standards

Dihydrocodeine: Extracted from DF118 tablets (Duncan Flockhart, London). 20 tablets (30 mg) were crushed and dissolved in H_2O (25 ml). The solution was filtered, basified (2 N NaOH) to pH 10.0 and extracted into chloroform (4 x 25 ml). The organic phase was separated, dried (anhydrous $MgSO_4$) and evaporated to dryness to yield the free base (520 mg). Morphine sulphate, strychnine, thebaine and atropine sulphate (Macfarlane Smith Ltd., Edinburgh).

Quinine sulphate - B.D.H. Ltd., Poole, Dorset

2.2.3 Solvents

Acetonitrile, methanol, chloroform, 1,2-dichloroethane, tetrahydrofuran, propan-2-ol, of HPLC grade and ethanol,butan-1-ol dioxan, toluene, diethylether and acetone of Laboratory Reagent grade, were supplied by Fisons Ltd., Loughborough, U.K.

Deuterated chloroform NMR solvent, containing 1% v/v tetramethylsilane (TMS) as internal standard, was purchased from Aldrich Chemical Co., Gillingham, Dorset.

The water used for the preparation of aqueous mobile phases was deionised and double distilled from a Fisons 'Fi-streem' all glass water still - deioniser.

2.2.4 Chemicals, reagents and stationary phases

2,2,2-trichloroethylchloroformate; Aldrich, Gillingham U.K. Sodium carbonate, sodium bicarbonate, zinc dust, magnesium sulphate (anhydrous), sodium hydroxide, monopotassium phosphate (KH_2PO_4), disodium phosphate ($Na_2HPO_4.2H_2O$), ammonium acetate (all of Analar grade) and triethylamine and diethylamine (Laboratory grade) were obtained from B.D.H. Ltd., Poole, U.K. Hydrochloric acid, sulphuric acid, glacial acetic acid, and pentane sulphonic acid pairing ion (HPLC grade) were obtained from Fisons, Loughborough, U.K.
Silica Gel $G.F._{254}$ TLC stationary phase, Partisil 5, Partisil 10 ODS-2, and ODS-Hypersil. HPLC stationary phases were purchased from Jones Chromatography, Glamorgan, U.K.

2.2.5 Equipment and instrumentation

Melting point apparatus: open ended capillary tube method, Gallenkamp, London.

NMR spectrometer; 100 MHz, Jeol JNM PS-100, Jeol, Tokyo.

Analytical balance; Shanton Unimatic, reading to 0.01 mg, Oertling Ltd., London.

Rotary Evaporator; Buchi, Fisons Scientific Apparatus

Ultra-violet/visible spectrophotometer; Perkin Elmer 550-s (Perkin Elmer Ltd., Slough,U.K.)

pH Meter; Pye Unicam Ltd. (Model PN 9418), standardised with buffer solutions of pH 4.0, 7.0 and 10.0. A constant pressure pump (Haskell model 27502, Olin Energy Systems, Sunderland, U.K.) was used for column packing.

HPLC instrumentation; The HPLC instrumentation was assembled from commercially available components. Mobile phase was pumped to the column by means of a constant flow liquid pump (either a Constametric III or a Milton Roy Minipump, LDC, Stone, UK). It was necessary to incorporate a pulse dampener when using the minipump.

The chromatography columns employed are listed in Table 2.1. Low dead volume Swagelok fittings (HETP) were used for the connections for the valve and detector.

Temperatures of the mobile phase reservoir, the column and injector were controlled by immersion in a heated water bath (Gallenkamp type 400-010). The output to the detector was recorded on a potentiometric chart recorder (Servogor 220, LDC Ltd.) and was also monitored by a Perkin Elmer Sigma X Data Station (Perkin Elmer,

Table 2.1. Summary of some of the properties of the packing materials and columns used in analytical HPLC

Column	Packing material	Particle Shape	Functionality	Column Length L (mm)	Column Internal Diamter dc (mm)	Nominal Particle Diameter dp (μm)	Porosity ε
A	Partisil 5	ITP (1)	Adsorption	150	4.6	5	0.68
B	Partisil 10 ODS-2	ITP	Reversed-phase	100	4.6	10	0.65
C	ODS-Hypersil	STP(2)	Reversed-phase	150	4.6	5	0.78

(1) ITP - irregular totally porous

(2) STP - Spherical totally porous

Slough, UK). Samples were introduced onto the column by means of a loop valve (Rheodyne 7125, Jones Chromatography).
The ultraviolet detectors were either a Cecil Model 212 variable wavelength (Cecil Instruments, Cambridge, UK) or a Pye Unicam LC-UV variable wavelength (Pye Unicam Ltd.).

2.3 Methods

2.3.1 Thin layer chromatography (TLC) methods

(a) Preparation of plates: 30 g silica gel GF_{254} was homogenised with 60 ml distilled water. The slurry was spread over ten 10 x 20 cm glass plates at a thickness of 0.25 mm. Plates were air dried and stored in a desiccator at 20°C. To ensure an even solvent front the outer 5 mm of adsorbent was stripped from both sides of each plate before use.

(b) Sample application: Samples of codeine and norcodeine base were applied to the plates in solutions of methanol (1 $\mu g\ ml^{-1}$) delivered from a 5 µl glass capillary applicator. The samples were applied in thin bands, 5 mm in length, positioned at 20 mm, above the bottom of the plate. Several layers were applied to each band, and the solvent evaporated from the plate between each application to prevent band broadening.

(c) Development: Freshly prepared mobile phase solvent (200 ml) was poured into a chromatography tank which was sealed. The

tank was placed in a heated water bath at a temperature of 25°C, and allowed to equilibriate for at least 4 hours. TLC plates were then lowered into the tank , which was resealed, and the solvent front was developed for 10 cm. at this temperature.

(d) Visualisation: Developed plates were initially examined under 254 nm ultraviolet light so that eluted components were detected by their fluorescence quenching. The plates were then sprayed with a 10% sulphuric acid solution and heated in an oven at 120°C for 15 minutes until visualised.

(e) Treatment of TLC results: Data relating to the TLC migration of sample components are expressed as hRf values:

$$hRf = \frac{\text{Distance travelled by component}^{A} \text{ (mm)} \times 100}{\text{Distance travelled by solvent front (mm)}} \quad 2.1$$

[A] Both upper and lower limits of each component spot are given.

2.3.2. High Performance Liquid Chromatography (HPLC) Methods

(a) Column packing

The columns used were slurry packed downwards in propan-2-ol using the apparatus described by Hamilton and Sewell[29]. Packing material (1.4 g per 100 mm x 5 mm i.d. of column) was dispersed in about 25 ml of solvent by immersion of the container in an ultrasonic bath. The slurry was then poured into the slurry

reservoir, and attached to the guard column and column (which contained propan-2-ol which was displaced as the column was filled). The pump was then charged to full pressure (5000 p.s.i.) and switched on, to pump the slurry through the column at maximum pressure. This was continued until the rate of flow from the column was reasonably constant, estimated by collecting the eluent over fixed time periods. At this stage the flow was switched off and the pressure allowed to fall to zero. After removing the column from the packing apparatus, a stainless steel mesh (2 μm) was placed on top, followed by a porous PTFE collar and the column head.

(b) Column testing

The quality of the column packing was tested according to the methods described by Bristow and Knox[77]. For straight phase columns, a test mixture of nitrobenzene(10^{-6}% v/v) and benzene (10^{-3}% v/v) in hexane was used, and the mobile phase was 1% v/v acetonitrile in hexane. For the reversed phase columns, the test solution was anisole (0.03% v/v) and phenetole (0.07% v/v) in methanol, and the mobile phase was methanol:water (70:30). Column efficiency, as described by h, (Eq. 1.5) for the columns A, B and C (Table 2.1) was found to be 3.6, 5.4 and 6.4 respectively.

(c) Preparation of mobile phases

(a) Non aqueous mobile phases were prepared by pipetting the appropriate amounts of each solvent into the reservoir and gently mixing them together.

(b) Aqueous mobile phases were prepared by pipetting the appropriate amounts of organic modifier, buffer (and pairing ion) solution into grade B volumetric flasks and adjusting to volume with double distilled-deionised water. All mobile phase solutions were passed through a 0.45 μm Millipore membrane filter under negative pressure and degassed with helium for 5 minutes before use.

2.3.3 Quantitative HPLC procedures

A series of analytical HPLC methods was developed for the separation of codeine and norcodeine. The methods involved one or other of the two procedures described below:-

(a) Internal standard procedure: To minimize errors due to sample preparation, apparatus and technique, a known compound of fixed concentration (the internal standard) was added to the unknown sample to give a separate peak in the chromatogram. The standard compound selected must be miscible with the sample, must be chemically stable, must not react with the sample components, and its retention time should be close to that of the sample component(s) of interest.

Calibration curves were obtained by chromatographing suitable volumes of calibration mixtures containing the compounds of interest with a constant concentration of the internal standard. Triplicate injections were made at each concentration. Peak heights and areas of the compounds of interest were determined,

and ratios were obtained from equations 2.2 and 2.3

$$\text{Peak Height Ratio} = \frac{\text{Height of analyte peak}}{\text{Height of internal standard peak}} \qquad 2.2$$

$$\text{Peak Area Ratio} = \frac{\text{Area of analyte peak}}{\text{Height of internal standard peak}} \qquad 2.3$$

Peak height and area ratios were plotted against concentration of compound, and each plot was subjected to regression analysis to determine the slope, intercept and correlation coefficient. The amount of compound (codeine or norcodeine) in a sample may be determined by division of the peak height/area ratio by the respective calibration slope. Between each set of assays, the calibration of the detector was checked by injecting a standard solution of codeine and norcodeine. If the peak height/area ratio deviated from the calibration curve by more than 5% the system was recalibrated.

(b) External standard procedure: This was adopted when it was not possible to 'fit in' a standard without overlapping another peak, which may often be the case in the analysis of samples from cell-free extract studies. A calibration plot was constructed by injecting a fixed volume of standard samples containing known weights (concentrations) of the compound(s) of interest. Plots of peak height and/or area versus weight (concentration) for each compound were obtained, and these checked for linearity by subjecting the data to linear regression analysis. The calibration

factor S (peak size/concentration) is equivalent to the slope, and the amount of the compound X (codeine or norcodeine) in an unknown sample was obtained from:-

$$\text{wt of x} = \left(\frac{\text{Peak height or area of x}}{S}\right) \qquad 2.4$$

To allow for possible changes in instrument parameters during analysis, the calibration factor (S) was checked at regular intervals. This was achieved by injecting standards of similar strength to the sample before and after each unknown sample.

(c) Precision of the detector response

This was determined for each developed HPLC assay by injecting sample solutions containing known concentrations of compound within the calibration range ten times. The mean standard deviation and coefficient of variation was determined for the responses obtained.

2.4 Experimental

2.4.1 Determination of the pKa's of codeine and norcodeine

The degree of ionization of a base (BH) can be expressed by acidic ionization constants:-

for $$BH^+ \rightleftharpoons B + H^+$$

$$ka = \frac{(H^+)\ (B)}{(BH^+)} \qquad 2.5$$

More conveniently the ionization constant is usually expressed as the pKa:-

$$pKa = pH + \log (BH^+) - \log (B) \qquad 2.6$$

Thus, the proportion of nonionized molecules of a base present in aqueous solution at a given pH can be calculated from:-

$$\% \text{ nonionised} = \frac{100}{1 + \text{antilog } (pH - pKa)} \qquad 2.7$$

pKa values for codeine and norcodeine were determined by potentiometric titration employing the method of Albert and Serjeant[78]. The temperature dial on the pH apparatus was set to the required temperature, and the glass electrode was standardized with buffer solutions at pH 4.0, 7.0 and 10.0. A solution of the substance was prepared (0.01 M) in double distilled-deionized water (50 ml) (codeine base 0.1497 g; norcodeine base 0.1427 g), and the solution brought to the required temperature. The pH of the solution was recorded initially. Then ten equal portions, each a tenth of an equivalent, of titrant (0.1 N HCl, f = 0.9074)

were added, and the pH recorded as soon as equilibrium was reached after each addition. Data from the titrations enabled the pKa's for codeine base and norcodeine base to be determined from equation 2.6. The results obtained were as follows:-

codeine base; pKa = 7.84 (±0.02) at 0.01 M and 25°C
(using 9 values in the set)
(literature value pKa 8.21[79])

norcodeine base; pKa = 8.79 (±0.02) at 0.01 M and 25°C
(using 9 values in the set)
(no lit. value found)

A prior knowledge of these pKa values was required in order to predict suitable conditions for the extraction and chromatographic studies described in this section.

2.4.2 U.V. absorption characteristics of codeine and norcodeine

The following experimental procedure was performed to find out if both codeine and norcodeine possess sufficient UV absorption characteristics to enable a UV detector to be employed in the subsequent HPLC studies.

Solutions of codeine (0.1 mg/ml) and norcodeine (0.05 mg/ml) were prepared in acetate (1%), acetic acid buffer at pH 5.5. The UV absorbance of each solution was recorded from 180 to 350 nm wavelength, using acetate buffer solution as a blank, and the results are illustrated in Figure 2.1.

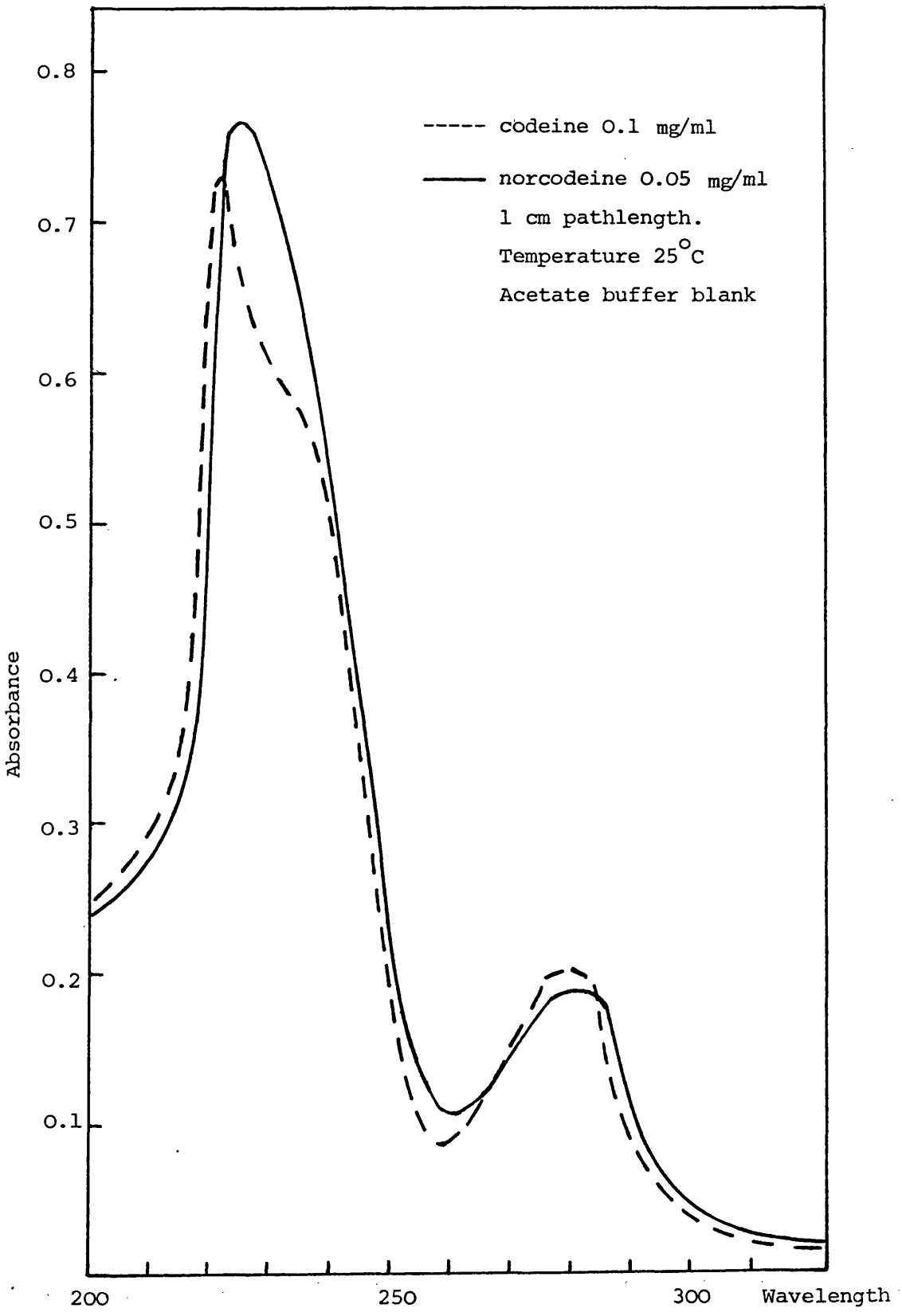


Fig. 2.1. The UV absorbance of codeine and norcodeine in acetate buffer against wavlength

Codeine and norcodeine produced absorption optima at about 285 nm wavelength ($\varepsilon^{1\%}_{1cm}$ codeine 31.5; $\varepsilon^{1\%}_{1cm}$ norcodeine 62.2), and 225 nm ($\varepsilon^{1\%}_{1cm}$ codeine 122; $\varepsilon^{1\%}_{1cm}$ norcodeine 252.7), and an absorption minimum in both cases at about 260 nm. The results indicate that the U.V. detector could be employed in the HPLC analysis of codeine and norcodeine. However, the wavelength of choice for an assay will depend on the UV absorption characteristics of the mobile phase, as well as those of codeine and norcodeine.

2.4.3 Straight phase HPLC development

a) Selection of the mobile phase

The primary step in the development of an HPLC separation is selection of the mobile phase. Thin Layer Chromatography (TLC) provides a rapid and economical method to achieve this.

A TLC method for the separation of codeine and norcodeine has been reported by Mulé[80], but the Rf values were not in the region 15 to 35, necessary for an adequate separation on a silica HPLC column. However, this system was used as a basis for the development of a suitable TLC system for the separation of codeine and norcodeine. The TLC procedure described in Section (2.3.1) was employed, and the solvent composition in the mobile phase modified to give Rf values between 15 and 35. Details of the original TLC system of Mulé and more suitable TLC systems developed are shown in Table 2.2.

Table 2.2. TLC retention data for codeine and norcodeine with various mobile phases on silica gel GF_{254} stationary phase

System	Mobile phase	hRf codeine	hRf norcodeine
1*	Ethanol (5); Dioxan (40), Benzene (40), NH_3 (5)	15-22	5-11
2	95% Ethanol (50), chloroform (45), Acetonitrile (5) 1% triethylamine **(in total)**	30-35	14-18
3	95% Ethanol (50), 1,2-dichloroethane(45) Acetonitrile (5) 1% triethylamine **(in total)**	32-36	14-16

Temperature 25°C

1* Mulé's original TLC system

Systems 2 and 3 (Table 2.2) gave Rf values in the desired region and were considered for further investigation in the straight phase HPLC analytical separation of codeine and norcodeine.

(b) Assay conditions

A Partisil 5 μm silica column (Column A, Table 2.1) was selected for its similar adsorbent characteristics to TLC silica gel. Some of the characteristics of Partisil 5 are listed in Table 2.1. The mobile phases developed from TLC in the previous section were investigated for their suitability in separating codeine and norcodeine, although the concentration of triethylamine was reduced from 1% v/v to 4×10^{-5} % v/v to minimise baseline noise.

Samples of codeine and norcodeine base dissolved in mobile phase (1 mg/ml) were injected onto the column, and the column capacity ratios (κ) and resolution (Rs) determined (from Equations 1.2 and 1.8 respectively) for each mobile phase. The results obtained are shown in Table 2.3. System 4 was selected for the quantitative determination of codeine and norcodeine because the best peak shapes were obtained with adequate retention and resolution of the components. A typical chromatogram is shown in Figure 2.2.

(c) Selection of internal standard

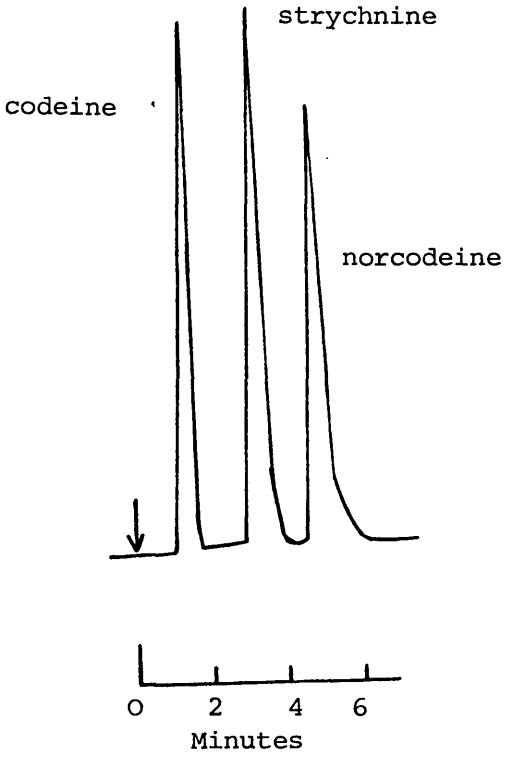
Samples of various alkaloids, selected as potential

Table 2.3. Retention data for codeine and norcodeine base on a Partisil 5 column with various mobile phases

System	Mobile phase	Column capacity ratio (κ)		Resolution
		Codeine	Norcodeine	(Rs.)
4	95% Ethanol (50), 1,2-dichloroethane (45), Acetonitrile (5) Triethylamine (4×10^{-5}%)	2.16	7.5	5.4
5	95% Ethanol (50), chloroform (45), Acetonitrile (5) Triethylamine (4×10^{-5}%)	2.0	9.15	7.3
6	Methanol (50), 1,2-dichloroethane (45), Acetonitrile (5) Triethylamine (4×10^{-5}%)	1.4	5.0	3.7
7	Methanol (50), Chloroform (45), Acetonitrile (5) Trimethylamine (4×10^{-5}%)	1.6	7.0	5.3

Temperature $25^{\circ}C$ Flow rate 1 ml min^{-1} UV detection 254 nm

Fig. 2.2. Example of straight phase HPLC chromatogram obtained in the assay of codeine and norcodeine



Analytical conditions

column;	Partisil 5 µm, 150 x 4.6 mm ID
mobile phase;	Ethanol (50), 1,2-dichloroethane (45), Acetonitrile (5), triethylamine (40 µl/100 ml).
temperature;	25°C
Flow rate;	2 ml/min
Detection;	UV 254
Injection volume	20 µl

standards, were dissolved in mobile phase (System 4) to give a concentration of 1 mg/ml, and each sample was injected onto the Partisil 5 column. From the retention data, the column capacity ratios (κ) were determined (Equation 2.2) for each alkaloid. The results obtained are given in Table 2.4.

Table 2.4 . HPLC retention data for codeine, norcodeine and a series of alkaloids

Standard	Retention time (minutes)	Column capacity ratio (κ)
Quinine	3.1	1.5
Thebaine	2.4	1.7
Codeine	3.1	2.1
Morphine	3.3	2.3
Dihydrocodeine	4.6	3.6
Strychnine	5.3	4.3
Norcodeine	8.1	7.1

The HPLC retention of strychnine (5.3 min) was equidistant between the retentions of codeine (3.1 min) and norcodeine (8.1 min) and was therefore selected as internal standard.

(d) Calibration procedure

Stock solutions containing codeine (100 mg) and norcodeine (100 mg) in mobile phase (System 4, Table 2.3) (50 ml) and strychnine, internal standard (75 mg) in mobile phase (25 ml),

were prepared. Sample volumes of the codeine and norcodeine stock solution from 1 ml to 8 ml were pipetted into volumetric flasks. Strychnine solution (1 ml) was also pipetted into each flask, and mobile phase to give a final volume of 10 ml in each case. The concentrations of codeine and norcodeine in each sample are listed in Table 2.5.

Table 2.5. Preparation of calibration samples for the straight phase assay of codeine and norcodeine

Sample volume (ml) pipetted	Final concentration (mg/ml)	
	codeine	norcodeine
1	0.2	0.2
2	0.4	0.4
3	0.6	0.6
4	0.8	0.8
5	1.0	1.0
6	1.2	1.2
7	1.4	1.4
8	1.6	1.6

Each sample was assayed in triplicate. Plots of the peak height, and peak height ratios of codeine and norcodeine against the concentration of the respective component in the calibration sample were constructed (Figure 2.3 and 2.4 respectively) and the results subjected to regression analysis. The data obtained are given in Table 2.6.

Figure 2.3. Peak height vs. concentration of codeine (o) and norcodeine (▲)

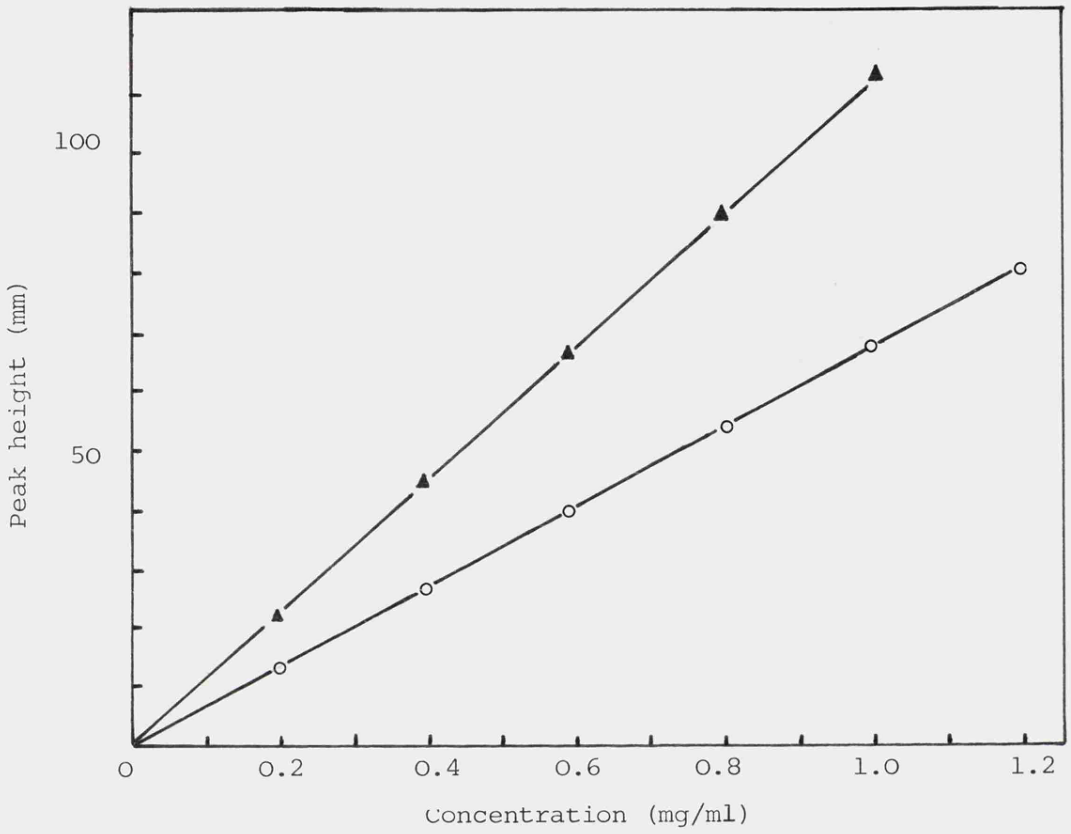


Figure 2.4. Plot of peak height ratio vs. concentration of codeine (o) and norcodeine (▲)

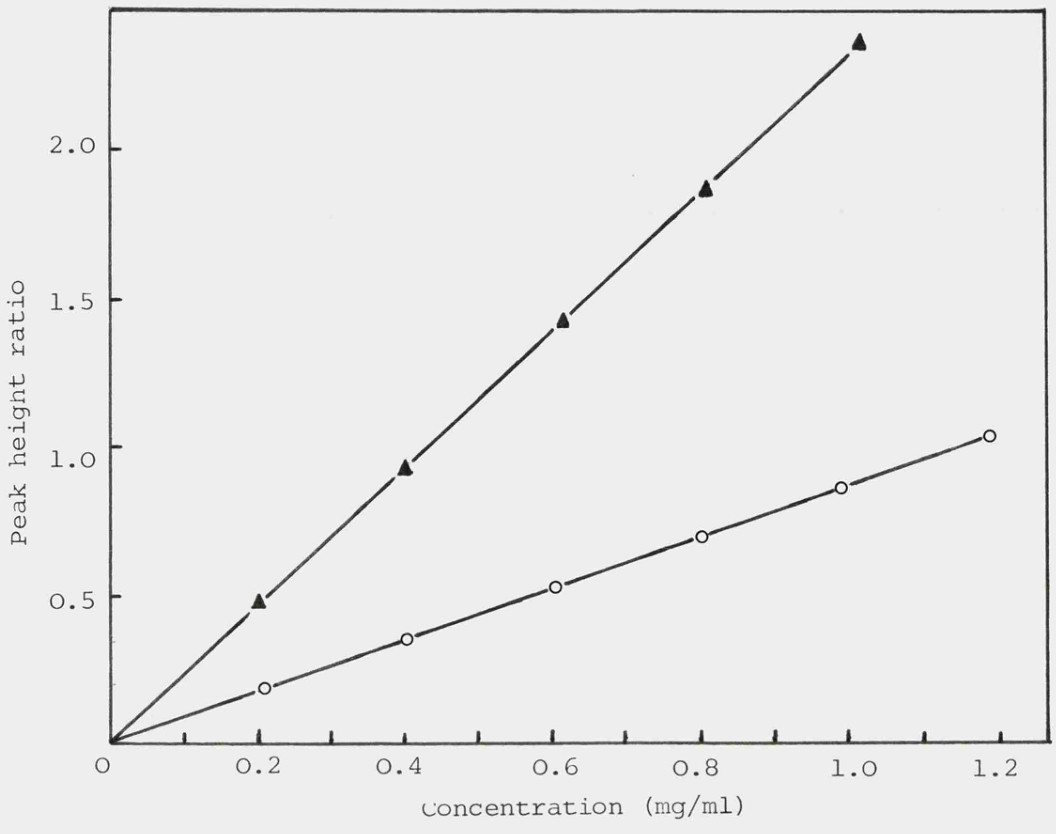


Table 2.6. Linear regression analysis of data obtained for the straight phase HPLC assay of codeine and norcodeine

	Peak height		Peak height ratio	
	Codeine	Norcodeine	Codeine	Norcodeine
Slope	63.1	113.5	0.825	2.37
Std. dev. of slope	0.629	1.119	0.00816	0.0252
Intercept	0.971	0.399	0.0138	0.0166
Std. dev. of intercept	0.512	0.766	0.0063	0.0080
Correlation co-efficient	0.9997	0.9998	0.9998	0.9998
% Coefficient of variation of slope	0.996	0.985	0.989	1.063

The results show that the HPLC system is suitable for the quantitative determination of codeine and norcodeine in the concentration range employed. However, the drugs have to be extracted from the transformation media prior to analysis, although this may not be necessary if a reversed phase system was employed.

2.4.4 Reversed phase HPLC - Partisil 10 ODS-2

(a) Selection of mobile phase

A series of organic modifiers listed in Table 2.7 was investigated. Each organic modifier was mixed with various ratios of acetate/acetic acid buffer (1%) at the arbitrary pH of 5.0. A mixture of codeine and norcodeine in mobile phase (1 mg/ml of each) was injected onto column B (Table 2.1) with each mobile

Table 2.7. Properties of the solvents investigated as organic modifier's in the separation of codeine and norcodeine by reversed-phase HPLC

From Snyders Classification of Solvents[82]

Solvent	p'	X_e	X_d	X_n
Methanol	5.1	0.48	0.22	0.31
Propan-1-ol	3.9	0.55	0.19	0.27
Tetrahydrofuran	4.0	0.38	0.20	0.42
Acetonitrile	5.8	0.31	0.27	0.42
Water (carrier)	10.2	-	-	-

phase in turn to find a system which separated the two compounds. The pH of the aqueous buffer, and the mobile phase flow rate, were later adjusted to optimise the separation.

The solvent systems which gave the best separation of codeine and norcodeine in terms of adequate resolution (Equation 1.8) in the least analysis time,and peaks exhibiting good column efficiencies (Equation 1.3) with the minimum of peak tailing are shown in Table 2.8.

Table 2.8. HPLC retention data for codeine and norcodeine on Partisil 10 ODS-2 column (B)

System	Mobile phase	pH of buffer	Flow rate ml/min	Codeine t_R(min)	Codeine κ	Norcodeine t_R(min)	Norcodeine κ
8	Methanol(30), Acetate (70) buffer	5.5	1.5	5.6	7.0	3.1	3.4
9	Acetonitrile(15), Acetate buffer (85)	5.0	1.5	5.5	8.8	3.1	4.5

(b) Assay conditions

System 8 (Table 2.8) was selected for the quantitative determination of codeine and norcodeine employing Column B. An ultraviolet variable wavelength spectrophotometer was employed for detection. To achieve the maximum sensitivity for the detection of codeine and norcodeine it was necessary to determine the wavelength at which these compounds have the highest absorbance, but

the other components in the mobile phase have the lowest.

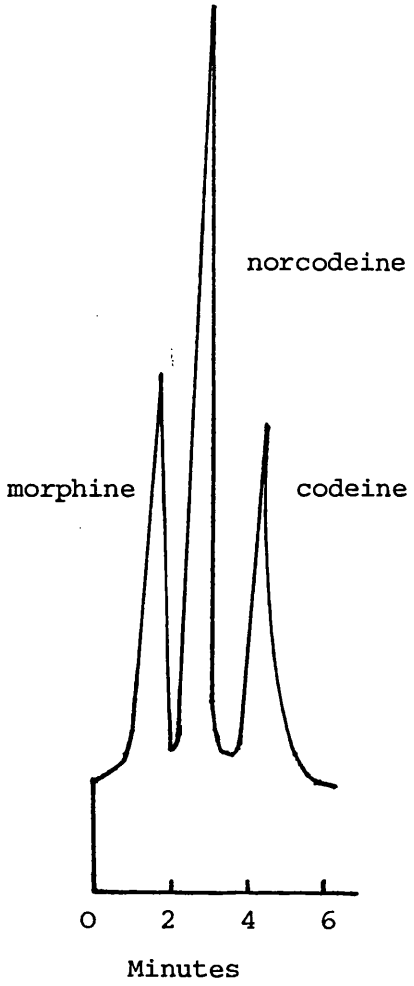
An ultraviolet scan of mobile phase was recorded against a distilled water blank from 180 to 350 nm. This was superimposed over the UV scans of codeine and norcodeine (Figure 2.1) determined in Section 2.4.2. The optimum wavelength was taken to be that where there is the maximum difference in UV absorbance between the compounds and mobile phase. This was observed to be 240 nm.

Morphine was selected as the internal standard. At a mobile phase flow rate of 1.5 ml/min the retention times of morphine , norcodeine and codeine were 1.6 mins, 3.0 mins and 5.6 mins respectively. A typical chromatogram is shown in Figure 2.5. with a summary of the operating conditions.

(c) Calibration procedure

Stock solutions containing codeine (100 mg) and norcodeine (100 mg) in mobile phase (System 8, 50 ml), and also a solution containing morphine standard (75 mg) in mobile phase (25 ml), were prepared. From these stock solutions a series of calibration solutions were prepared as shown in Table 2.5. Morphine internal standard was added to each calibration solution to a final concentration of 0.3 mg /ml. Each calibration solution was assayed in triplicate by the reversed phase HPLC procedure. Regression analysis of the peak height/area against concentration data gave the results shown in Table 2.9.

Fig. 2.5. Example of HPLC chromatogram obtained in the assay of codeine and norcodeine employing Partisil 10 ODS-2



Analytical conditions

Column;	Partisil 10 ODS-2, 100 x 4.6 mm ID
Mobile phase;	Methanol (30), Acetate buffer (70), pH 5.5
Temperature;	25°C
Flow rate;	1.5 ml min^{-1}
Detection;	UV 240 nm
Injection volume;	100 µl

Table 2.9. Linear regression analysis of data obtained for the Partisil 10 ODS-2 assay of codeine and norcodeine

	Peak height ratio		Peak area ratio	
	Codeine	Norcodeine	Codeine	Norcodeine
Slope	0.569	2.380	2.069	2.93
Std.Dev. of slope	0.0103	0.0206	0.0031	0.0228
Intercept	0.0186	0.037	0.0720	-0.051
Std.Dev.of intercept	0.0085	0.0161	0.0344	0.0017
Correlation coefficient	0.9993	0.9998	0.9995	0.9998
% Coeff. of variation of the slope	1.807	0.865	0.149	0.778

These results demonstrated that the HPLC assay was suitable for the quantitative determination of codeine and norcodeine in the concentration range likely to be encountered in general transformation studies[18]. However, for enzyme kinetic studies lower concentrations would be encountered, so it was necessary to determine the linear range at the detection limits of the assay.

(d) Linear range and detection limits

The linear range of the detector response was determined by chromatographing successive dilutions of the codeine and norcodeine stock solution with mobile phase. Morphine internal standard was added to each solution and the peak height/area responses determined in each case. It was necessary to adjust the detector sensitivity between dilutions to obtain a measurable

response on the chart recorder, but the corrected response to internal standard was found to be constant at each sensitivity confirming correct dilution procedure.

The detection limit was considered to be the concentration at which the response was not less than 10% of a full-scale deflection, at the maximum sensitivity of the UV detector. This was found to be equivalent to 10^{-3} mg ml, for both codeine and norcodeine in mobile phase 8. Regression analysis carried out on plots of peak height and area ratios of codeine and norcodeine against their concentration in the samples are given in Table 2.10 and suggest a linear range from 1 x 10^{-3} mg ml^{-1} to 1 mg ml^{-1} for each compound.

Table 2.10. Linear regression analysis of data obtained for the linear range of the detector response for codeine and norcodeine with the Partisil 10 ODS-2 Column

	Peak height ratio		Peak area ratio	
	Codeine	Norcodeine	Codeine	Norcodeine
Slope	0.936	1.984	3.444	3.358
Std.dev. of slope	0.0074	0.0058	0.0479	0.0274
Intercept	-0.0076	-0.0135	-0.0048	-0.0024
Std.Dev. of intercept	0.0034	-0.0026	0.0021	0.0011
Correlation coefficient	0.9998	0.9999	0.9996	0.9998
% Coeff. of variation of the slope	0.791	0.292	1.391	0.816

(e) Precision of the detector response

A solution of codeine and norcodeine in mobile phase 8 was chromatographed ten times using the conditions described in Figure 2.5. The concentration of solution selected (0.05 mg ml^{-1}) was approximately from the middle of the linear range tested. The results obtained are given in Table 2.11.

Table 2.11. Precision results for the Partisil 10 ODS-2 HPLC assay of codeine and norcodeine in mobile phase

For 10 injections	Peak height ratio		Peak area ratio	
	Codeine	Norcodeine	Codeine	Norcodeine
Mean	0.58	2.35	2.07	2.87
Standard deviation	0.011	0.0458	0.0418	0.0614
% Coefficient of variation ±	1.89	1.95	2.02	2.14
% range of error ±	3.02	3.116	3.228	3.419

Hence for assays determined employing duplicate injections the % range of error = $\pm \frac{t.cv}{\sqrt{N}}$ where t is the 't' test value at 95% confidence and N is the number of replicate injections per sample (2).

The HPLC assay was repeated with calibration solutions prepared in transformation media to check for any interference from components in the transformation mixture. Unfortunately the resulting chromatograms revealed a variably large solvent front which occluded the standard peak and prevented the peak

height/area ratios from being determined. The regression analysis for the peak heights and areas against concentration, in the absence of internal standard, gave the results shown in Table 2.12.

Table 2.12. Linear regression analysis of data obtained for the Partisil 10 ODS-2 assay of codeine and norcodeine in transformation media

	Peak height		Peak area	
	Codeine	Norcodeine	Codeine	Norcodeine
Slope	1824	3865	80.42	78.44
Std.dev. of slope	6.58	33.08	0.64	0.516
Intercept	-0.249	0.215	0.0063	0.068
Std.dev. of intercept	0.089	0.076	0.0031	0.023
Correlation coefficient	0.9999	0.9998	0.9999	0.9999
% coeff of variation of slope	0.36	0.85	0.796	0.658

A sample of codeine and norcodeine in transformation media (0.05 mg ml^{-1}) chromatographed ten times gave the results in Table 2.13.

Table 2.13. Precision results for the Partisil 10 ODS-2 HPLC assay of codeine and norcodeine in transformation media

For 10 injections	Peak height (mm)		Peak area (mm^2)	
	Codeine	Norcodeine	Codeine	Norcodeine
Mean	15.6	24.4	4.23	3.87
Standard deviation	0.318	0.548	0.0914	0.086
% Coefficient of variation ±	2.04	2.24	2.16	2.22
% range of error ±	3.26	3.58	3.45	3.55

These results indicate that the peak heights and areas could be employed in an external standard procedure (Section 2.3.3(b)) for the determination of codeine and norcodeine directly from transformation samples. However, it was thought that this assay would not be sufficiently sensitive for the determination of these drugs at the concentrations encountered in enzyme kinetic studies.

2.4.5 Reversed phase HPLC assay - ODS-Hypersil, 5 µm

(a) Assay conditions

A mobile phase was selected for Column C by employing the systematic procedure established in the previous section for Column B. This consisted of acetonitrile organic modifier 16% v/v with phosphate buffer at pH 6.5. Also included was an ion-pairing agent, pentane sulphonic acid (5 mM) to enhance the retention

of codeine and norcodeine. A summary of the operating conditions employed and a typical chromatogram of the separation are shown in Figure 2.6. For these conditions the retention times of norcodeine and codeine were 8.0 and 12.0 minutes respectively.

(b) Calibration procedure

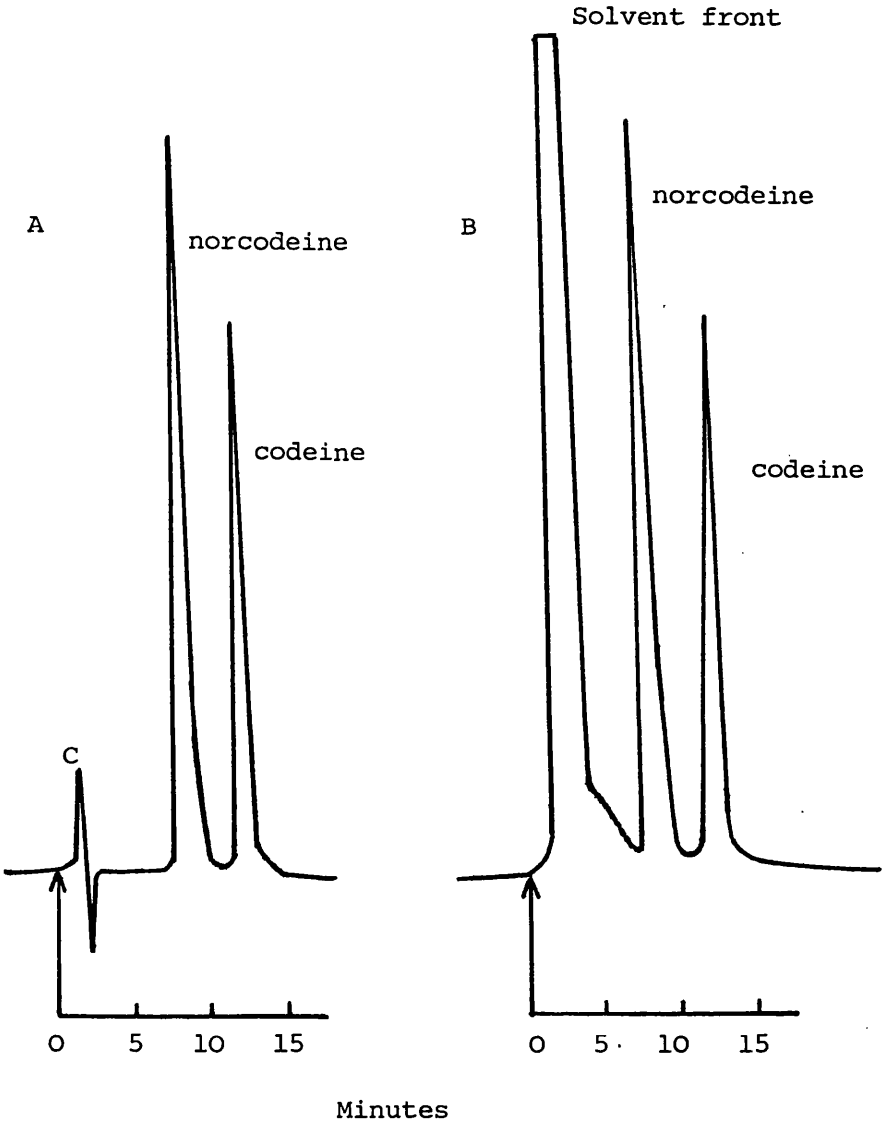
The external standard procedure described (Section 2.3.3(b)) was employed. A calibration plot was constructed to check the linearity of the detector response, and the limits of detection for codeine and norcodeine, using the procedure described previously for the Partisil 10 ODS-2 system, except that in this assay no internal standard was included.

The detection limit was 1×10^{-4} mg/ml for both codeine and norcodeine, and the linear range of the detector response, calculated from the peak height and peak area measurements, was 1×10^{-4} mg/ml to 25×10^{-2} mg/ml supported by the regression analysis given in Table 2.14 .

(c) Precision of the detector response

Sample solutions containing codeine and norcodeine (0.05 mg/ml) in mobile phase and transformation media respectively were each injected ten times onto column C. Table 2.15 shows the results obtained indicating that the precision of the detector response was unaffected by components in the transformation mixture.

Fig. 2.6. Example of chromatogram obtained with ODS-Hypersil HPLC assay for codeine and norcodeine in mobile phase (A) and in transformation media (B)



Analytical conditions

Column;	ODS-Hypersil 5 µm, 150 x 4.6 mm I.D.
Mobile phase;	Acetonitrile (16), Phosphate buffer (84), pH 6.5 Pentane sulphonic acid pairing ion (5 mM)
Temperature;	$25^{o}C$
Flow rate;	1.5 ml min^{-1}
Detection;	UV 240 nm

Table 2.14. Linear regression analysis of data obtained for the linear range of the detector response for codeine and norcodeine with the ODS Hypersil Column

	Peak height (mm)		Peak area (mm^2)	
	Codeine	Norcodeine	Codeine	Norcodeine
Slope	9.100	16.5	4192	8037
Std.dev. of slope	0.3605	0.223	95.57	100.46
Intercept	-0.15	-0.250	-0.64	-1.35
Std.Dev. of intercept	0.059	0.113	0.028	0.54
Correlation coefficient	0.9977	0.9997	0.9996	0.9995
% coeff. of variation of slope ±	3.96	1.35	2.28	1.25

Table 2.15. Precision results for the ODS-Hypersil HPLC assay of codeine and norcodeine in mobile phase (A) and in transformation media (B) based on peak height measurements

For 10 injections	A		B	
	Codeine	Norcodeine	Codeine	Norcodeine
Mean	41.6	64.2	40.8	63.7
Standard deviation	0.765	1.129	0.799	1.159
% Coeff. of variation ±	1.84	1.76	1.96	1.82
% range of error ±	2.94	2.81	3.13	2.91

A sample in mobile phase B sample in transformation media

Part B. Recovery and Purification Methods

2.5 Materials

2.5.1 Column packing materials and HPLC stationary phases

Amberlite XAD-2, XAD-4 and XAD-7 non-ionic resins - BDH Ltd. Lichroprep silica, 15 - 25 μm particle size (Merck) and Lichroprep RP18 silica, 25 - 40 μm particle size (Merck) were purchased from Jones Chromatography, Glamorgan.

2.5.2 Equipment and instrumentation

Phase separating filter paper; Fisons Ltd., U.K. 7 litre laboratory fermenter, 2000 series, - LH Engineering Co. Ltd., Stoke Poges was used to produce 7 litre batches of transformation material. The design, construction and operation of this fermenter were described by Sewell[18].
GLC oven; Perkin Elmer Type F11 (Perkin Elmer Ltd., Slough, UK) was employed for the attempted reactivation of preparative HPLC columns.

(a) Instrumentation for group extractions with Partisil 10 ODS

The sample to be extracted was pumped to the column by means of a constant pressure liquid pump (2 ml volume Haskell type). The column was stainless steel (100 mm x 5 mm i.d.) packed with 1.02 g of Partisil 10 ODS-2. It was connected to the pump by means of stainless steel tubing and standard Swagelok fittings (HETP). A graduated glass vessel and timer

were used to collect and measure the flow rate of sample through the column. Experimental temperatures were maintained by immersing the column and sample reservoir in a thermostatically heated water bath.

(b) Instrumentation for group extractions with Amberlite resins

For the preliminary investigations a glass column (100 mm x 5 mm i.d.), with tap, was plugged at the tap end, with a stainless steel filter (mesh size 5 μm) and glass wool, and filled with 1 g of accurately weighed resin material. For the preparative extraction studies a glass column (500 mm x 25 mm i.d.) was plugged in a similar manner, and filled with 100 g of the selected resin material. All the resin materials were washed thoroughly with methanol (HPLC grade) followed by distilled water, before they were poured into the columns as a slurry in distilled water. The sample to be extracted was pumped from the reservoir to the top of the column by means of a peristaltic pump (Loughborough Glass Co. Ltd.). Samples were eluted from the column under gravity flow, at a rate controlled by the tap at the base of the column, and collected in a graduated glass vessel.

(c) Preparative HPLC instrumentation

All the preparative HPLC studies were carried out using

an 830 Preparative High Performance Liquid Chromatograph (Dupont, USA). This consisted of the following essential components contained in a complete unit:-

(a) A 70 ml stroke capacity constant pressure pump; to pump mobile phase from a 2 litre capacity stainless steel reservoir to the column;

(b) Loop valve; Rhehodyne 7125, with sample loops of either 2 ml or 10 ml volume, connected to the injection valve via 1/16" O.D. stainless steel tubing;

(c) Detector; Ultraviolet variable wavelength spectrophotometer (Dupont ,USA) fitted with a short path length preparative flow cell;

(d) Fraction collection valve assembly; to direct the flow of mobile phase from the column either back to the reservoir, to drain, or through one of three fraction outlets to a collecting vessel.

(e) A thermostatically controlled oven; which housed the preparative HPLC column, and injection port assembly. The oven, with the aid of a mobile phase preheater, connected between the pump outlet and injection valve inlet, were used to control the operating temperature.

A schematic diagram of how these components were connected is illustrated in Figure 2.7. The preparative chromatography columns were stainless steel and of dimensions listed in Table 2.16.

Fig. 2.7. Generalised scheme of the preparative liquid chromatograph system

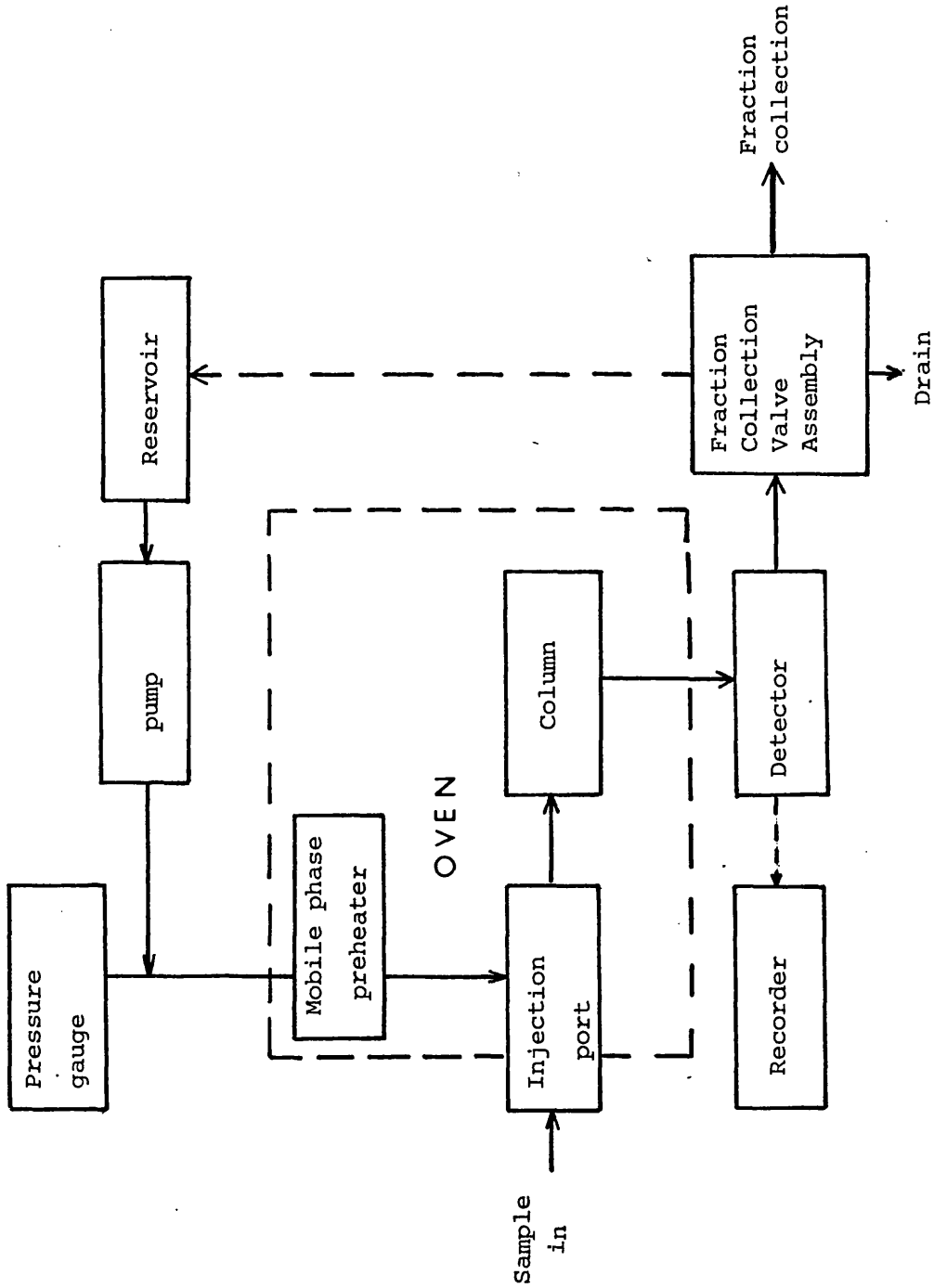


Table 2.16. Summary of some properties of the packing materials and columns used in the preparative HPLC studies

Column	Packing material	Functionality	Column length L (mm)	Column internal diameter dc (mm)	Particle size dp (μm)	Amount of packing material in column (g)
D	LiChroprep Si60	Adsorptive	250	22	15-25	52.96
E	LiChroprep RP-18	Reversed-phase-partition	250	8	25-40	7.66
F	LiChroprep RP-18	Reversed-phase-partition	500	22	25-40	105.70

2.6 Methods

2.6.1 Batch extraction procedure with Partisil 10 ODS-2 and Amberlite resins

A solution of codeine and norcodeine base (10 mg of each) in the medium to be extracted (100 ml), was adjusted to pH by the dropwise addition of acid (2N HCl) or base (2N NaOH). The sample solution was passed through a 0.45 μm membrane filter (Millipore Type HA) to remove particulate matter. The filtrate sample was poured into the reservoir, and pumped through the column (Partisil 10 ODS-2 or Amberlite resin) at a controlled flow rate and temperature. When the reservoir was almost empty, distilled water (25 ml) was added and pumped through the system to flush remaining sample solution from the pump and connections to the column. The column and pump were disconnected and acetone (200 ml) passed through the pump to clear it out. The column and pump were then reconnected, and an eluting solvent pumped through the column at a controlled flow rate. The organic eluent was collected, dried (anhydrous $MgSO_4$) and filtered, and the solvent was then evaporated in a rotary evaporator under reduced pressure over a water bath at 50°C. On cooling, the residue was dissolved in mobile phase (5 ml), containing strychnine internal standard (0.3 mg/ml), and the amount of test compound (codeine, norcodeine) recovered in the organic phase was determined by straight phase HPLC (Section 2.4.3). The amount of test compound extracted per gram of adsorbent material was expressed by:-

$$\frac{\text{Amount recovered in organic phase (mg)}}{\text{Amount of adsorbent material (g)}} \qquad 2.8$$

and the extraction efficiency was expressed by:-

$$\frac{\text{Amount recovered in organic phase}}{\text{Amount added to medium}} \times 100 \quad 2.9$$

At least 10 column volumes of 0.1 N HCl followed by 10 column volumes of distilled water were pumped through the column before the next sample solution was introduced.

2.6.2 Preparative HPLC methods

(a) Column packing and testing

The columns used were slurry packed downwards using the technique described in Section 2.3.2(a). Packing material (0.7 g per c.c. internal volume of the column) was dispersed in chloroform (4); methanol (1) to produce the slurry. At the completion of column packing, the excess packing material was collected, dried and weighed, and subtracted from the initial weight of stationary phase used to determine the weight of stationary phase in the column. These values, and details of the preparative columns packed are listed in Table 2.16.

All the columns packed were subjected to the column tests described in Section 2.3.2(b) to evaluate the quality of the packing.

(b) Injection procedure

The sample to be separated was dissolved in the minimal quantity of mobile phase, and filtered through a 0.45 μm Millipore

(Type HA) membrane filter to remove particular matter. The resulting solution was injected into the sample loop, of 2 ml or 10 ml capacity, as appropriate. Mobile phase was pumped through the column at a predetermined rate, and when a peak was detected, the eluate was collected from one of the fraction collection outlets. The mobile phase flow rate was determined by collecting the column eluate in a graduated vessel over a known time interval.

2.7 Experimental

2.7.1 The group extraction of codeine and norcodeine from transformation media employing Partisil 10 ODS-2 HPLC packing material

In order to determine the parameters which provide efficient and consistent extraction of codeine and norcodeine from transformation mixtures , systematic studies were carried out on transformation media to which codeine and norcodeine were added. The effects of various parameters on the capacity of Partisil 10 ODS-2 reversed phase material to quantitatively extract codeine and norcodeine from transformation media were investigated. These parameters included; pH of the transformation media, the temperature at which the extraction was achieved, the flow rate of transformation media through the column, the polarity of the eluting solvent, and the addition of methanol to the transformation media before extraction.

For 1.02 g of Partisil 10 ODS-2 material in the column extraction efficiencies were determined (from Equation 2.9) for the extraction of codeine and norcodeine (10 mg of each) from transformation media for each of the parameters investigated. The results are shown in Table 2.17. Acetone was selected as the solvent to elute the compounds from the column since it was found to be far more effective than 1,2-dichloroethane and 1,2-dichloroethane (80), propan-2-ol (20).

The optimum recovery of codeine and norcodeine occurred when the pH of the medium was 8.5, the temperature was 4°C, the flow rate of medium through the column was 5 ml/min; acetone was the eluting solvent and 5% v/v methanol was added to the medium. Extraction efficiencies for codeine and norcodeine under these conditions were 70% and 68% respectively, or 6.86 and 6.66 mg/g of test compound per gram of packing material.

2.7.2 Group extraction of codeine and norcodeine from transformation mixtures employing Amberlite resins (1 gram scale)

Amberlite XAD-2, XAD-4 and XAD-7 resins were examined for their capacity to quantitatively extract codeine and norcodeine from transformation media as a function of pH of the media. Codeine and norcodeine sample solutions in media, adjusted to the selected pH, were passed through the columns containing each type of resin material (1.0 g). Acetone was employed to elute the drugs from the column in the desorption stage. The flow rate of acetone, and the sample media, was controlled at 1 ml/min, and the

Table 2.17. The effects of various parameters on the capacity of Partisil 10 ODS-2 material to quantitatively extract codeine and norcodeine from transformation media

Experimental temperature °C	pH of medium	Flow rate of medium through column ml/min	Addition of methanol to medium % v/v	Extraction efficiency % codeine	Extraction efficiency % norcodeine
25	6.5 *	5.0	-	37	35
25	7.5 *	5.0	-	60	55
25	8.5 *	5.0	-	62.5	60
25	9.5 *	5.0	-	59.5	58
25	8.5	5.0	5 *	68.5	65
25	8.5	5.0	10 *	66.5	64
25	8.5	2.5 *	5	65	62
25	8.5	7.5 *	5	66	61
4 *	8.5	5.0	5	70	68
35 *	8.5	5.0	5	56	53

* indicates which parameter is varied

experimental temperature was maintained at 25°C in each case.

Extraction efficiencies were determined using Equation 2.9 and the values obtained are listed in Table 2.18. The maximum extraction efficiency was achieved with XAD-4 resin, at a pH of 9.5 for the extraction of norcodeine (95%) and at a pH of 8.5 for the extraction of codeine (78%). Under the same conditions values of 12.6 mg/g and 9.9 mg/g were obtained for the amount of norcodeine and codeine extracted per gram of resin. This was a considerable improvement compared to the Partisil 10 ODS-2 material. On the basis of these findings the XAD-4 material was employed for the preparative scale extraction of codeine and norcodeine from transformation media.

2.7.3 Group extraction of codeine and norcodeine - Amberlite XAD-4 resin (100 g)

In the previous section the extraction efficiency of XAD-4 resin (1.0 g) was determined by spiking transformation media with a known quantity of codeine and norcodeine at a fixed concentration. However, in microbial transformation studies, variable concentrations of substrate and the transformation products are encountered, with the product concentration in the transformation mixture increasing at the expense of the substrate. Therefore the recovery of codeine and norcodeine was determined over a range of concentrations employing the XAD-4 resin column method. A recovery curve was constructed where the amount recovered by extraction was plotted on the ordinate against the

Table 2.18. The effect of pH on the extraction of codeine and norcodeine from transformation media by XAD-2, XAD-4 and XAD-7 Amberlite resins

Resin material	pH of media	Extraction efficiency (%)	
		codeine	norcodeine
XAD-2	8.5	71	74
	9.5	69	87
	10.5	67	86
XAD-4	8.5	78	79
	9.5	76	95
	10.5	75	94
XAD-7	8.5	30	24
	9.5	30	25
	10.5	35	30

Temperature, 25°C

Flow rate; 1 ml/min

Eluting solvent; Acetone

amount added to the growth medium (abscissa) and the slope of the plot was determined. Since the theoretical maximum recovery would have a slope of unity the % recovery is given by:-

% recovery = slope of recovery plot x 100%

The extraction of codeine and norcodeine was determined simultaneously from transformation medium containing glucose (1% w/v) and casein hydrolysate (1% w/v). Codeine and norcodeine base were added to the above growth medium (250 ml) in each of five flasks to give the ratios given in Table 2.19.

Table 2.19. Ratios of codeine to norcodeine in samples of transformation medium (250 ml) for their group extraction by Amberlite XAD-4 resin (100 g)

Solution	Weight of compound added	
	Codeine base(mg)	Norcodeine base (mg)
1	100	-
2	70	30
3	50	50
4	30	70
5	-	100

The contents of each flask were basified to pH 9.5 by the dropwise addition of 2N NaOH, and passed through the XAD-4 (100 g)

resin column at a controlled flow rate of 2 ml min^{-1}. Acetone was passed through the column to desorb the drugs, and the eluate collected, dried (anhydrous $MgSO_4$), filtered (Whatman No. 1 filter paper) and evaporated to dryness on a rotary evaporator. The residue was reconstituted in mobile phase, and the codeine and norcodeine concentration determined by the straight phase HPLC procedure (Section 2.4.3). The total amounts of codeine and norcodeine recovered from each flask were plotted against the respective amounts of codeine and norcodeine added, and then subjected to linear regression analysis. The results obtained are given in Table 2.20.

Table 2.20. The % recovery of codeine and norcodeine from transformation media using the XAD-4 resin procedure, and regression analysis of the data obtained.

Flask	codeine recovered		norcodeine recovered	
	mg	%	mg	%
1	84.9	84.9	-	-
2	58.51	83.6	28.95	96.5
3	41.25	82.5	47.87	95.75
4	24.93	83.1	68.11	97.3
5	-	-	97.7	97.7

	CODEINE	NORCODEINE
Slope	0.858	0.985
Std. dev. of slope	0.01026	0.0079
Intercept	-1.264	-0.916
Std.dev. of intercept	0.674	0.434
Correlation coeff.	0.9999	0.9999
% coeff. of variation of slope ±	1.196	0.802

The linearity of the recovery plot demonstrated that the extraction efficiency is independent of codeine and norcodeine concentration over the range examined. Extraction efficiencies of 85.8% and 98.5% respectively, indicated the suitability of the procedure for the extraction of both compounds from transformation media.

2.7.4 Straight phase preparative HPLC

(a) Selection of chromatographic conditions

A stainless steel column (250 mm x 22 mm internal diameter) packed with LiChroprep Si60 silica (15 - 25 µm particle size) was employed for these studies (Column D, Table 2.16). Generally, the easiest and most economical way to a preparative separation is a scale-up from the analytical separation[81]. It uses less solvent and time than does the direct use of a preparative column. Therefore, the mobile phase developed in Section 2.4.3 for the analytical separation of codeine and norcodeine on the Partisil 5 column, consisting of: 95% ethanol (50), 1,2-dichloroethane (45), acetonitrile (5) and triethylamine (4×10^{-5}% v/v), was initially employed. Detection of the compounds was by UV. It was necessary to employ the UV detector at a wavelength exhibiting the lowest sensitivity to codeine and norcodeine, so that the peak response remained on the chart recorder. This was determined to be 260 nm for codeine and norcodeine from the UV scans of the drugs (Figure 2.1).

(b) Determination of the optimum flow rate

Codeine and norcodeine base were dissolved in mobile phase to produce a solution containing 4 mg/ml of each compound. Samples of this solution (2 ml) were injected onto the preparative HPLC column at mobile phase flow rates from 15 to 40 ml/min. From the chromatograms produced at each mobile phase flow rate, values were determined for the average plate number (N), the reduced plate height (h), the linear flow rate (u) and the reduced velocity (v) from Equations 1.3, 1.5, 1.6 and 1.7 respectively. These data were used to construct plots of h against v (Figure 2.8(a)) and $(v/h)^{\frac{1}{2}}$ against v, (Figure 2.8(b)) for both codeine and norcodeine. The most efficient flow rate for the separation of codeine and norcodeine was then determined from these plots, for the stated chromatographic conditions. In the h against v plot the most efficient flow rate was obtained when h was at a minimum[46], and in the $(v/h)^{\frac{1}{2}}$ against v plot when (v/h) was at a maximum[82], On this basis the optimum flow rate for the separation of codeine and norcodeine on the LiChroprep Si60 column was found to be 25 ml/min.

(c) Determination of the column loading capacity at the optimum flow rate

The loading capacity of the preparative column was determined by injecting successively increasing quantities of sample until the components of interest remain just sufficiently pure for the purpose of separation. The loading capacity was then estimated from Equation 2.10

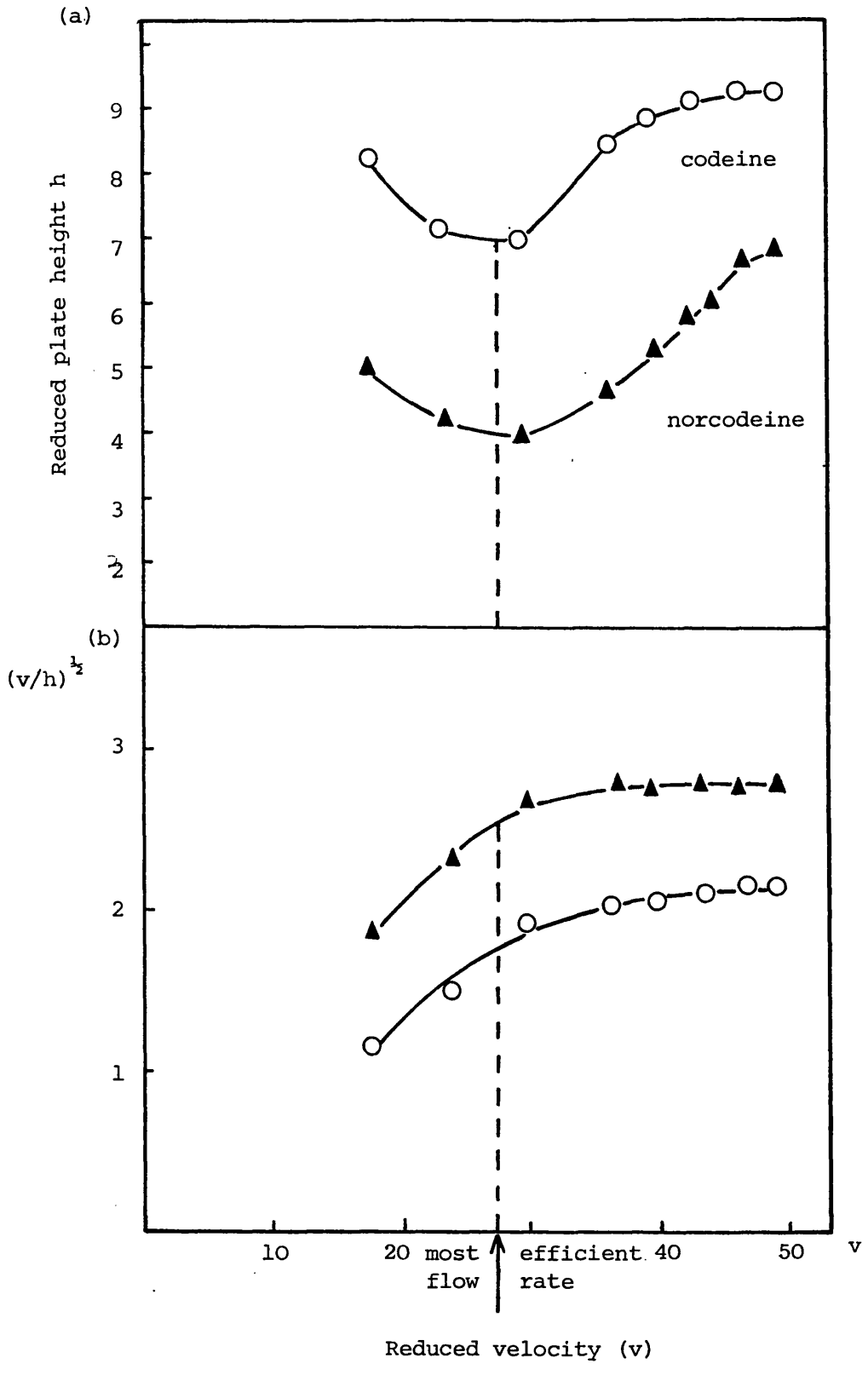


Fig. 2.8. Determination of the most efficient flow rate for the separation of codeine (O) and norcodeine (▲) on Lichroprep Si 60 column

$$\text{Loading capacity (mg/g)} = \frac{\text{weight of sample separated (mg)}}{\text{weight of column materials (g)}} \quad .. 2.10$$

Equal quantities of codeine and norcodeine base were dissolved in mobile phase to give a range of increasing concentrations from 2 mg/ml to 100 mg/ml. Each solution was filtered (Millipore 0.45 μm) and samples (2 ml) were injected onto the LiChroprep Si-60 column. As the components eluted from the column, fractions of the peaks were collected, and analysed for purity using the Partisil 5 straight phase HPLC assay (Section 2.4.3). The following parameters were determined from the resulting chromatograms for both codeine and norcodeine:- the column capacity ratio at the peak front (κ_1) and the peak tail (κ_2), the average plate number measured at 10% of the peak height ($N_{0.1}$), the resolution (Rs) between codeine and norcodeine peaks, and the symmetry factor ($A_{10\%}$) for each peak. These data were used to construct plots of κ_1 and κ_2, $N_{0.1}$, Rs and $A_{10\%}$ respectively, against the quantity of codeine and norcodeine loaded onto the column. The plots are shown in Figures 2.9 to 2.12. The effect of loading on the peak shape of the codeine and norcodeine peaks is illustrated in Figure 2.13.

It was concluded from the column loading studies that a maximum of 100 mg each of codeine and norcodeine could be separated and resolved on the LiChroprep Si-60 column. Analysis of the component fractions collected by analytical HPLC established that both codeine and norcodeine were of 100% purity at this loading. The loading capacity for column D, determined from Equation 2.10, was 3.76 mg of codeine and norcodeine per gram of stationary phase.

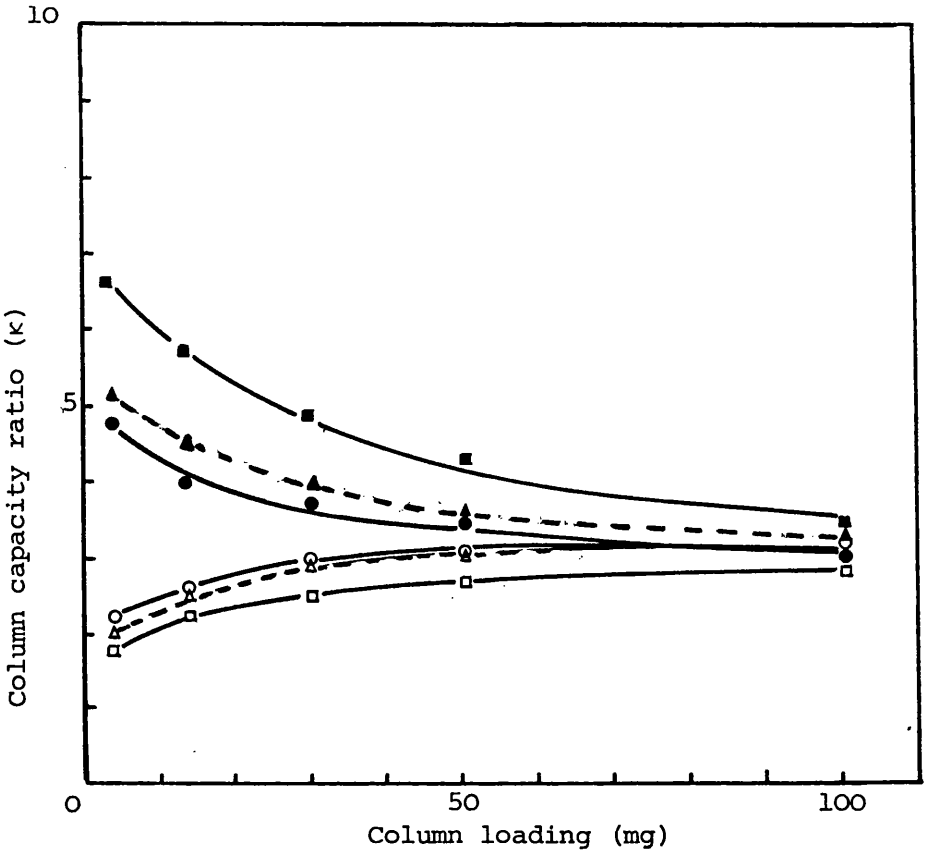


Figure 2.9. Column capacity ratios (κ) for the peak tail of codeine on a used o , regenerated Δ and repacked column □ and the peak front of norcodeine on a used ● , regenerated ▲ , and repacked ■ column with column loading

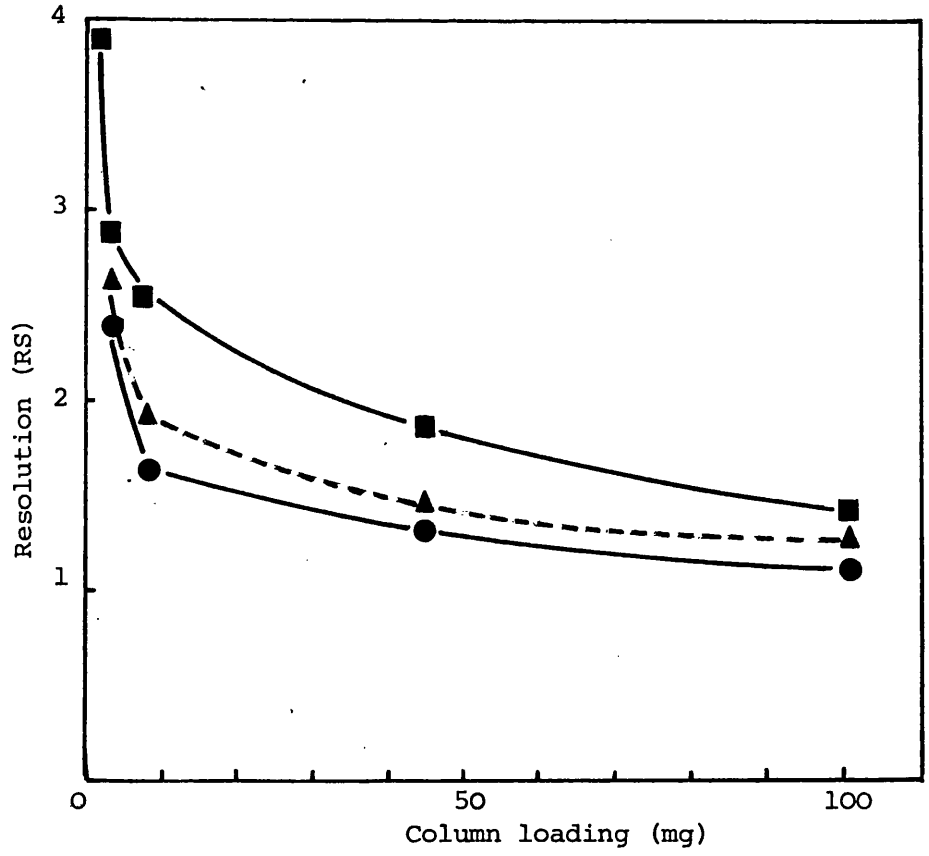


Figure 2.10. Resolution (RS) between codeine and norcodeine on a used (●), regenerated (▲) and a repacked column (■) with column loading.

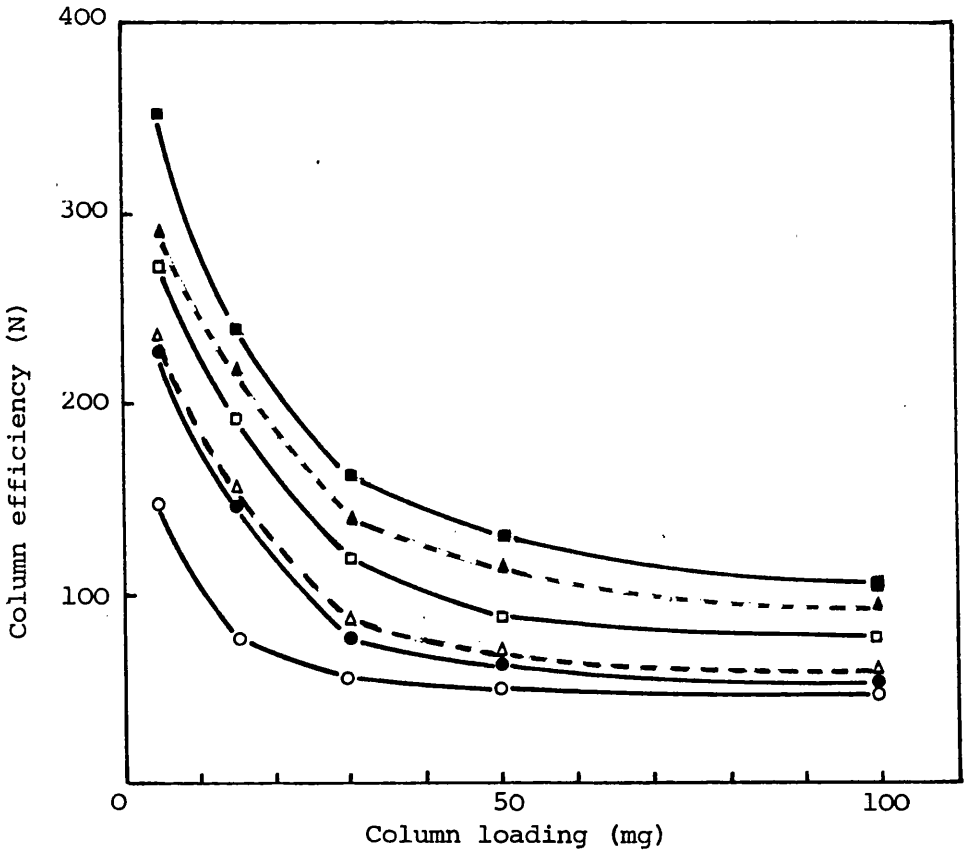


Figure 2.11. Column efficiencies (N) of codeine on a used ○, regenerated △ and repacked □ column, and norcodeine on a used ●, regenerated ▲ and repacked ■ column, with column loading

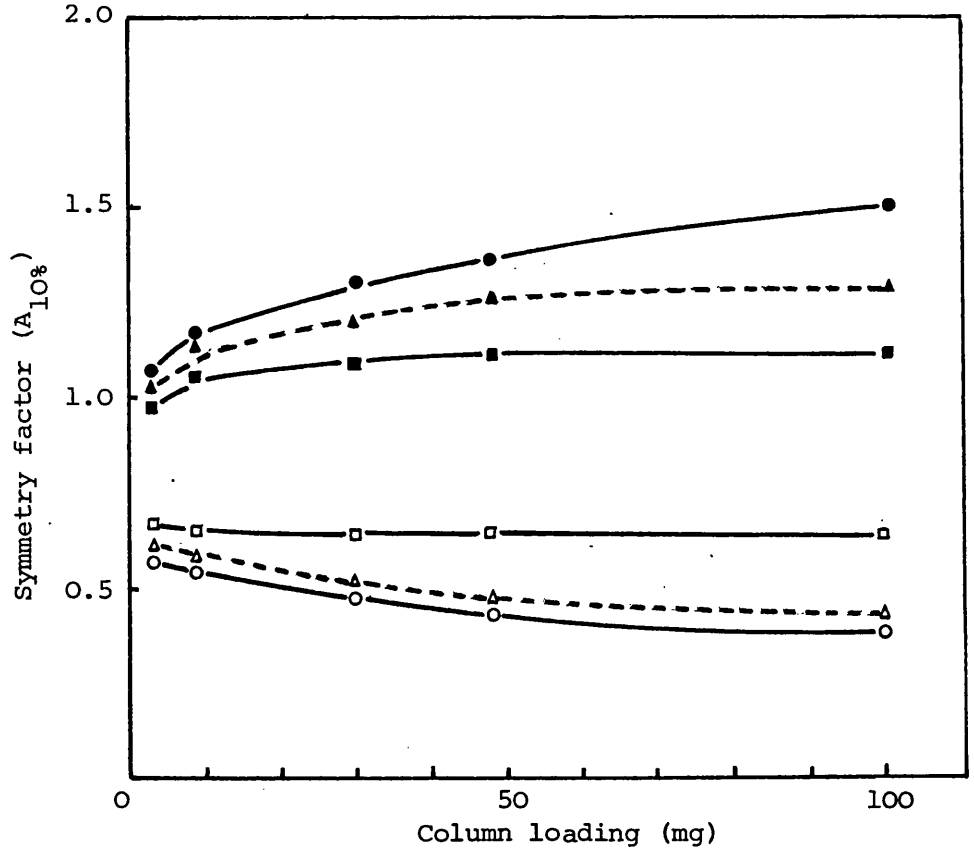
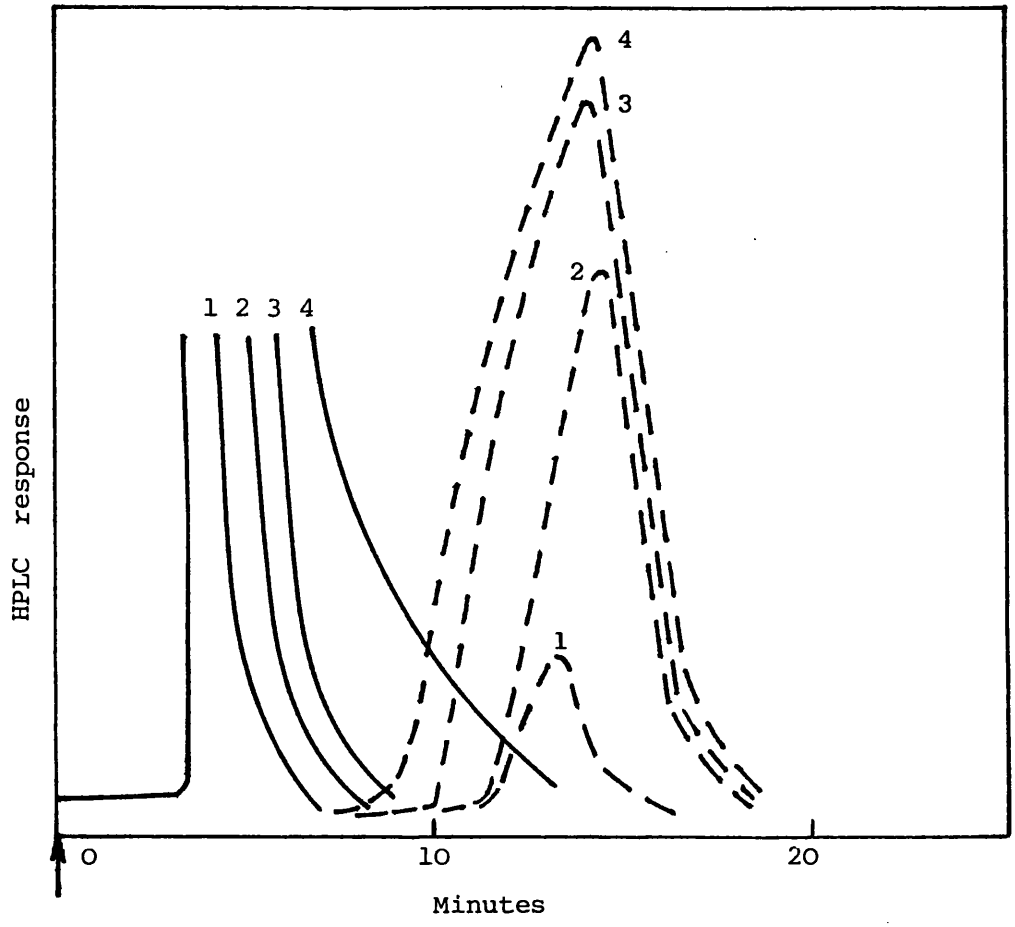


Figure 2.12. Peak symmetry ($A_{10\%}$) of codeine peaks on a used ○, regenerated △ and repacked column □, and norcodeine peaks on a used ●, regenerated ▲, and repacked column ■ with column loading.

Fig. 2.13. The effect of loading on the peak shape of codeine and norcodeine

codeine	1. 4 mg	Norcodeine	1. 4 mg
———	2. 50 mg	— — — →	2. 50 mg
	3. 100 mg		3. 100 mg
	4. 150 mg		4. 150 mg



Chromatographic conditions

Column; Lichroprep Si 60, 15-25 μm, 250 mm x 22 mm I.D.

Mobile phase; 95% ethanol (50); 1,2-dichloroethane (45); acetonitrile (5); Triethylamine (4×10^{-5} % v/v)

Temperature; 25°C

Flow rate; 25 ml/min

Detection; UV 260 nm

However, it was envisaged that the loading capacity could be increased by 'cleaning' the column, and so a column regeneration procedure was attempted.

2.7.5 Attempted regeneration of the straight phase column

The following column regeneration procedure was attempted with column D (Table 2.16).

The organic solvents, methanol (15 column volumes), acetone (15 column volumes) and tetrahydrofuran (15 column volumes), were pumped through column D in turn. The column was then removed from the HPLC pump, and attached to a nitrogen cylinder. A slow bleed (5-10 ml min^{-1}) of nitrogen gas was passed through the column for 4 hours. The column was then placed inside a G.C. oven, and attached to the oven by the inlet end, leaving the exit end of the column open. A slow bleed of nitrogen gas was passed through the column, as the oven temperature was raised slowly at a rate of $2^{o}C$ per hour until a temperature of $150^{o}C$ was reached. The column was baked for 15 hours at $150^{o}C$ with the nitrogen gas still passing through. Then the GC oven was turned off and allowed to cool down naturally, without opening the oven door. The column was then disconnected from the GC oven, and attached to an HPLC pump. Hexane was pumped through the column at 2 ml/min for 2 hours. At least 10 column volumes of tetrahydrofuran, acetone and methanol were pumped through the column in turn at 2 ml/min. Finally, the desired mobile phase was pumped through, and allowed to equilibrate

with the column for one hour.

The loading capacity for the reactivated column (D) was determined at the optimum flow rate (as described in Section 2.7.4(c)) and the values obtained for the parameters κ_1, κ_2, $N_{0.1}$, Rs and $A_{10\%}$ used to reconstruct the plots of κ_1 and κ_2, $N_{0.1}$, Rs and $A_{10\%}$ respectively against column loading of codeine and norcodeine, shown in Figures 2.9 to 2.12 by the broken line.

These results show a marginal improvement in column performance with regard to the separation of codeine and norcodeine, but the loading capacity of 3.86 mg g^{-1} was not a significant improvement. Subsequently, column D was repacked with the same stationary phase, and the column loading studies with codeine and norcodeine repeated once more (see Figures 2.9 to 2.12). As a consequence, a mixture containing 135 mg of both codeine and norcodeine were separated with 100% purity, giving a loading capacity of 5.12 mg codeine and norcodeine per gram of stationary phase. These results were compared with values obtained for reversed-phase materials in the following section.

2.7.6 Reversed-phase preparative HPLC

(a) Chromatographic conditions

To save analysis time and solvent usage the codeine-norcodeine separation was initially achieved on an analytical scale employing column E (Table 2.16). The ratio of methanol

(organic modifier) to acetate (1% w/v), acetic acid buffer was varied to obtain a separation, and this was optimised by final adjustment to the pH of the buffer. This occurred with a mobile phase of methanol (40), acetate (1% w/v), acetic acid buffer (60), pH 6.5 and a flow rate of 1 ml/min. So these conditions were used in an attempt to separate codeine and norcodeine on a preparative HPLC column (500 mm x 22 mm i.d.) packed with a similar stationary phase material (column F, Table 2.16). It was found that the optimum separation of the components on column F was obtained with a mobile phase consisting of methanol (40), acetate (1% w/v), acetic acid buffer (60), pH 7.0 and a flow rate of 20 ml/min. When the acetate buffer was replaced by phosphate buffer at the same pH, the resolution between codeine and norcodeine was improved, but the peak shapes were not as good. Typical chromatograms obtained with these systems are shown in Figure 2.14.

(b) Loading capacity of column F

For these studies a 10 ml sample loop was employed. The detector wavelength was adjusted to its lowest sensitivity for codeine and norcodeine (260nm) as shown in Figure 2.1. Mobile phase B in Figure 2.14 was selected at a flow rate of 20 ml/min. Equal quantities of codeine and norcodeine salts were dissolved in the minimum of mobile phase, filtered (0.45 μm Millipore Type HA), and injected onto column F. The peak fractions were collected and analysed for purity using the Partisil 10 ODS-2 reversed-phase assay (Section 2.4.4). Successive samples were injected containing increasing

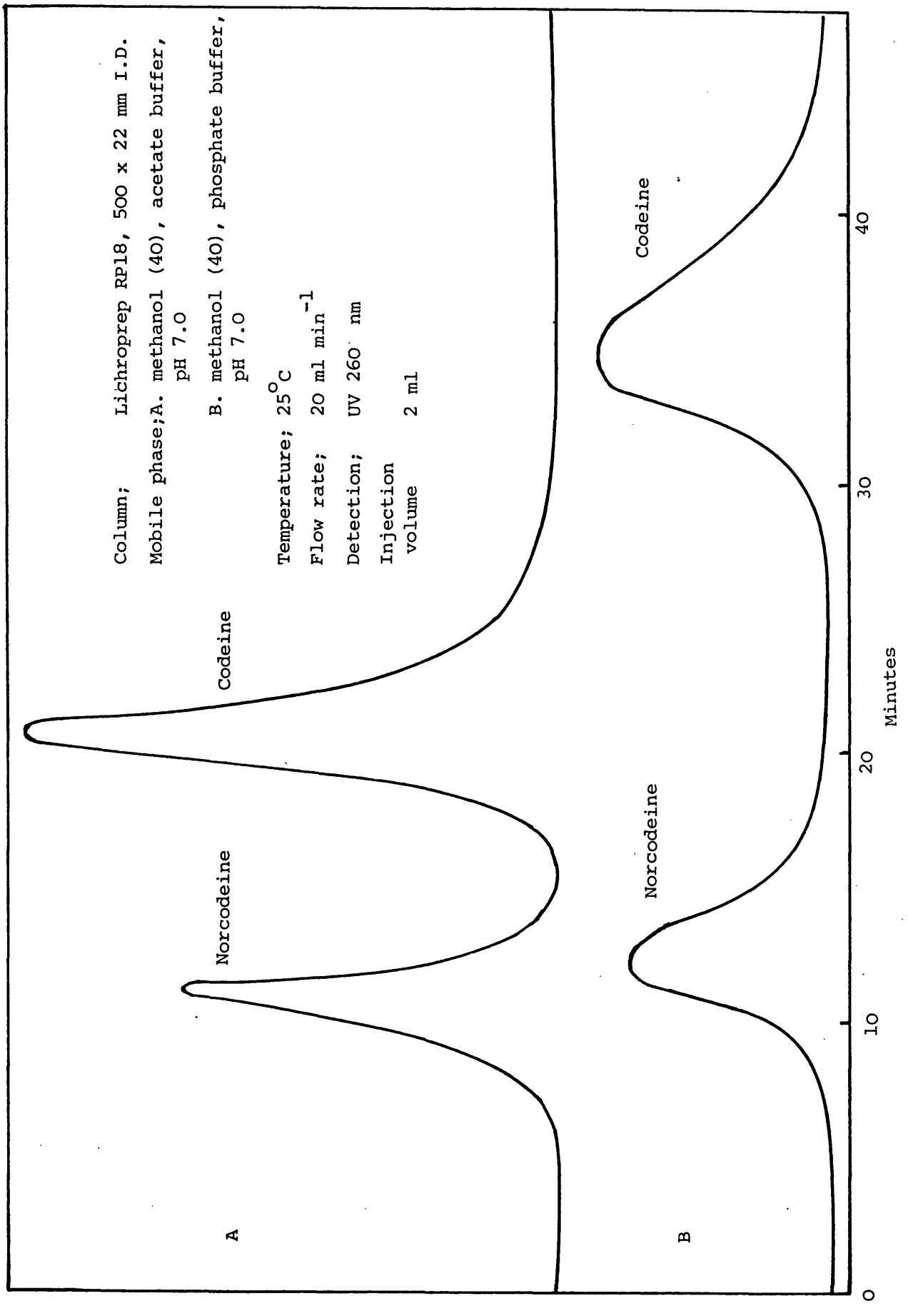


Fig. 2.14. The effect of the buffer solution on the separation of codeine-norcodeine mixture (2 mg ml^{-1})

quantities of test compounds until a separation was no longer achieved, and the purity of the eluted compounds was less than 95%.

It was concluded that a maximum quantity of 1002 mg (equivalent to 552 mg codeine base and 450 mg norcodeine base) in a 10 ml injection sample could be separated and resolved from column F, with a purity of 98%. This corresponds to a loading capacity (from Equation 2.10) of 9.48 mg test compounds per gram of stationary phase.

2.7.7 The combined group and fractionating extraction of codeine and norcodeine from 7 litre fermenter samples

6,900 ml of transformation mixture was taken from the 7 litre fermenter after ten days incubation with *Cunninghamella bainieri* (C43) and basified to pH 9.5 by the dropwise addition of 5N NaOH. The fungal cells were filtered (Whatman No. 1 filter paper) under negative pressure and washed with distilled water. An aliquot (1 ml) was taken for HPLC analysis (Partisil 10 ODS-2 method, section 2.4.4) to determine the initial quantity of codeine and norcodeine present in the mixture. The mixture was then divided into approximately three equal portions. Each portion was extracted in turn employing the Amberlite XAD-4 resin (100 g) column procedure described in Section 2.7.3, and acetone to finally desorb the alkaloids from the column. The acetone eluates were combined, dried (anhydrous $MgSO_4$), filtered (Whatman No. 1 filter paper)

and divided into two equal fractions. Each fraction was evaporated to dryness on a rotary evaporator to leave two residues. These were dissolved in the minimum of the respective mobile phases, and the codeine and norcodeine in one of the samples was separated by straight phase preparative HPLC (Section 2.7.4), and in the other sample, by reversed-phase preparative HPLC. Pure codeine and norcodeine were recovered from the straight phase mobile phase fractions by evaporating the organic solvent to dryness. Recovery from the reversed phase mobile phase was achieved by removing the organic component by evaporation (Rotary evaporator), basifying to pH 9.5, and re-extracting the drugs using the XAD-4 (100 g) column. A schematic diagram to illustrate the total extraction scheme is shown in Figure 2.15. At every stage of the proceedings aliquots of sample were taken for analytical HPLC analysis to determine the recovery and purity of the drugs. These data are shown in Table 2.21 and are discussed on page 126.

Figure 2.15. The total extraction and isolation of codeine and norcodeine from 7 litre transformation mixtures

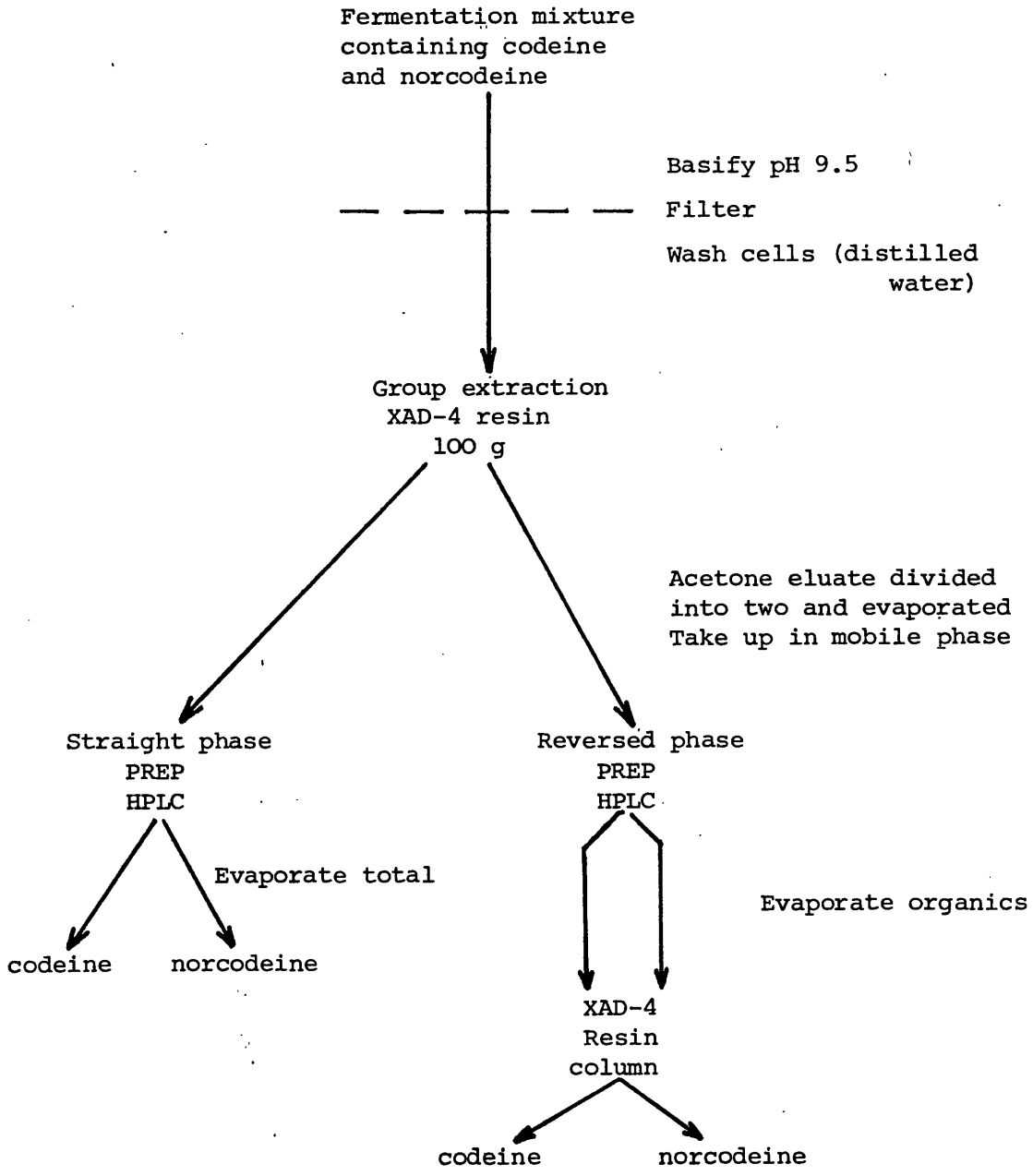


Table 2.21. The extraction of codeine and norcodeine from a 7 litre batch transformation mixture

Stage of procedure	Quantity recovered (grams)		% recovery	
	Codeine	Norcodeine	Codeine	Norcodeine
Starting transformation mixture 6900 ml	1.9044	0.8942	100	100
XAD-4 resin	1.6592	0.8246	87.12	92.2
Sample divided into 2 equal fractions	0.8296 + 0.8296	0.4123 + 0.4123	87.12	92.2
Reversed phase PREP HPLC	0.7946	0.3973	80.95	88.86
XAD-4 resin to recovery drugs	0.6785 98.6% (purity)	0.3608 97.5%	71.25	80.69
Straight phase PREP HPLC	0.7542 96.7% (purity)	0.3755 97.2%	79.20	83.97

2.8 Discussion

2.8.1 Analytical HPLC methods

Codeine and norcodeine both contain basic groups which ionise in aqueous solution depending on the pH. The pKa values of 7.84 and 8.79 (± 0.02) were obtained for the potentiometric titration of 0.01 M solutions of codeine and norcodeine respectively at 25°C. These pKa values were required in order to calculate the extent of ionization of these compounds in aqueous media (from Equation 2.7) at a given pH. This acquired data was employed, for example, to predict the optimum pH for the extraction of codeine and norcodeine from the media, as generally only uncharged species tend to partition into the organic phase. Alternatively, the degree of ion pairing in reversed phase HPLC depended on the pH of the aqueous mobile phase, so that ion pairing only occurred if the solutes were in their charged or ionized state.

In order to study codeine and norcodeine by HPLC a suitable method for their detection was required. The ultraviolet (UV) detector is probably the most widely used of detectors[29], and is employable if the substances of interest absorb radiation in the UV region of the spectrum. The UV scans shown in Figure 2.1 demonstrated adequate UV adsorptions for both drugs. The UV detector was therefore employed for all the HPLC studies described in this chapter.

The codeine and norcodeine separation was initially achieved by T.L.C. This is an acceptable procedure, because the mode of

separation in both TLC and straight phase HPLC is based on liquid-solid adsorption, and there is often a direct correlation between the TLC separation, and the separation on a silica HPLC column[83]. This was found to be true for a series of alkaloids investigated as potential internal standards for the Partisil 5 HPLC assay (Section 2.4.3(c)). For each of the alkaloids investigated the column capacity ratio (κ) was determined on column A. From published TLC retention data[84] on silica gel TLC plates impregnated with a base, and ethanol as mobile phase, a series of Rm values was also obtained for the alkaloids investigated, where :-

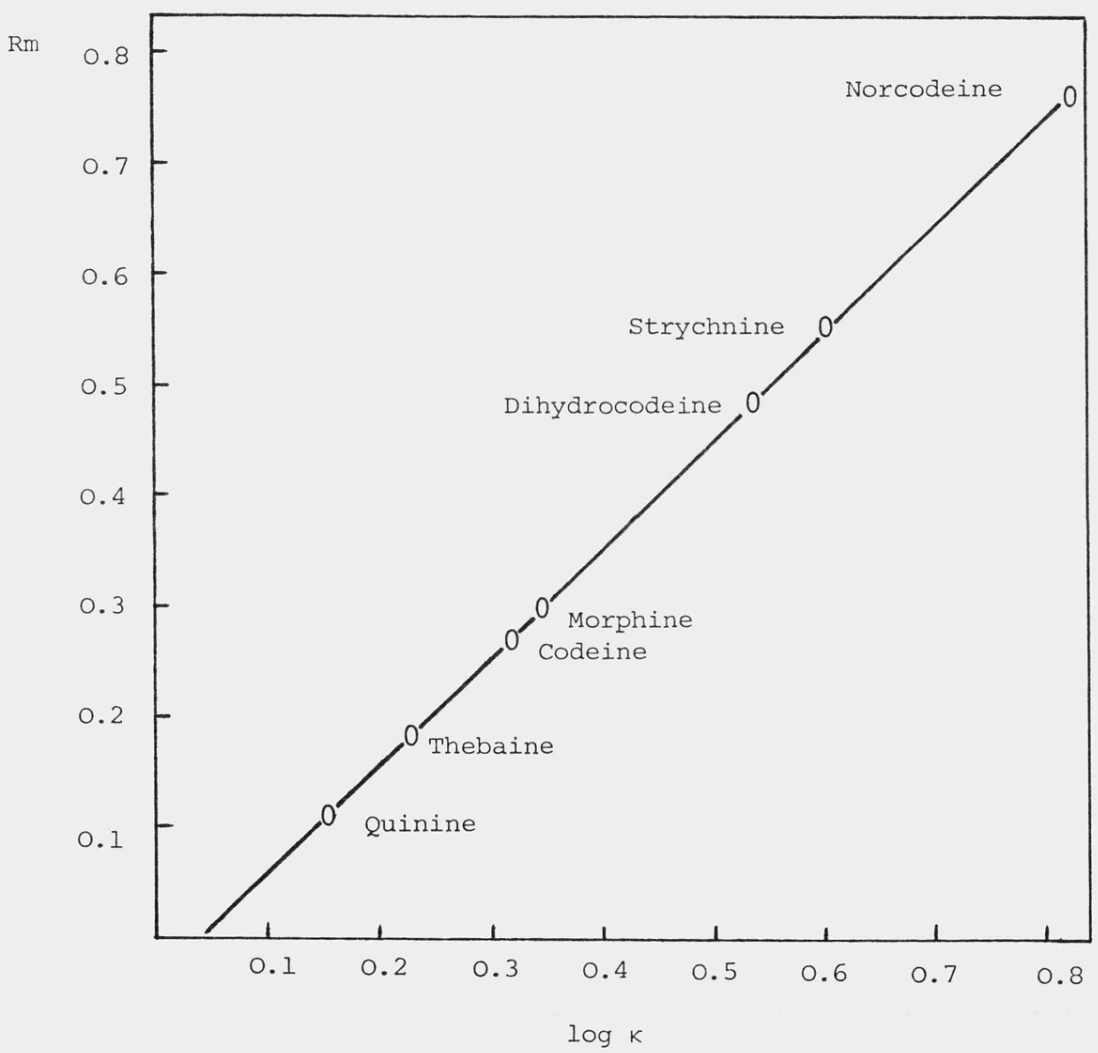
$$Rm = \log \left(\frac{1}{Rf} - 1 \right) \qquad 2.11$$

The Rm value is a measure of the TLC retention of the solute. The graph of log κ against Rm (Figure 2.16) was linear suggesting that there was a direct correlation between the TLC retention of each alkaloid and HPLC retention.

The distance travelled by a compound on a TLC plate will depend on the relative affinity of the compound for the stationary and mobile phase, and the choice of solvent(s) in the mobile phase will determine the retention value. Attempts have been made to quantify the strength of solvents by giving them numerical values based on their dielectric constants[85], their solubility[86] or their polarity[87]. Snyder[88] introduced a solvent characterization parameter (P') which distinguishes between solvent strength (the ability to dissolve more polar

Fig. 2.16. Relationship between HPLC retention data and published TLC retention data for a series of alkaloids.

(for details, see text)



molecules preferentially) and solvent selectivity (the ability to dissolve one compound selectively as opposed to another where the polarities are similar). Solvents were classified into groups on the basis that similar classes of solvent will behave similarly, and each solvent was quantified to give even greater precision in the characterization of solvents used in chromatography. Solvents were therefore selected from Snyders classification tables[88] to modify the composition of mobile phase and give an adequate separation of codeine and norcodeine by TLC (Table 2.2).

From the TLC data a series of mobile phases were developed (Table 2.3) to achieve a separation on the Partisil 5 μm column (A). These were essentially of the same composition as the TLC mobile phases developed previously except for the concentration of base (triethylamine), which was reduced to minimise baseline noise. Strychnine was found to be the most suitable internal standard, of the alkaloids investigated, because its retention was equidistant between the retentions of codeine and norcodeine. An HPLC assay was performed to quantitatively determine codeine and norcodeine in a concentration range likely to be encountered in transformation studies[18], employing strychnine as the internal standard. Although this procedure produced excellent linear regression results (Table 2.6), it suffered from a disadvantage that the drugs have to be extracted from the transformation media prior to their analysis. Sewell[18] has reported a possible extraction procedure employing 1,2-dichloroethane at pH 10.5, with extraction efficiencies of 98 and 96%

for codeine and norcodeine respectively, but this presents an extra time consuming step in the analysis. However, it was envisaged that this problem may be overcome by employing a reversed phase HPLC method, and analysing the samples directly from transformation mixtures.

In the initial studies column B (Table 2.1) containing Partisil 10 ODS-2 was selected for its high carbon loading of 15%, which was thought to be an advantage for the increased retention of codeine and norcodeine in complex biological samples such as transformation media.

In reversed phase chromatography the mobile phase is usually a combination of water, and a water miscible organic solvent (organic modifier). Solute retention can be modified in various ways, for example, by manipulation of the water to organic modifier ratio , by changing the pH if the solute is ionizable, or by selecting a different organic modifier. Unlike the straight phase system, it would have been uneconomical to employ TLC to find a suitable mobile phase, because reversed phase TLC plates are relatively expensive.

A systemetic procedure was adopted to select the most suitable mobile phase. First, an organic modifier was sought which separated the compounds of interest. The separation was then optimised by adjusting the organic modifier to water ratio and/or the pH of the mobile phase. The organic modifier was selected from Snyder's classification of solvents[88], where the

relative ability of each solvent to interact as a proton donor, proton acceptor or dipole is measured by Xd, Xe and Xn values respectively. Solvents with the same solvent strength P', but different values of Xd, Xe and Xn contributions will show selectivity changes,which may lead to a better separation of the sample components. The organic modifiers investigated are listed in Table 2.7, with their particular solvent strengths and selectivity values. As a result of this investigation, the mobile phase consisting of methanol (30): acetate buffer (70), pH 5.5 was selected for the HPLC assay of codeine and norcodeine employing column B. With this system, detection limits of 1×10^{-3} mg ml^{-1} were obtained for both codeine and norcodeine, and the detector response exhibited a linear range from 1×10^{-3} mg ml^{-1} to 1 mg ml^{-1}, with the precision of response shown in Table 2.11. These results demonstrated the suitability of the Partisil 10 ODS-2 method for the quantitative determination of codeine and norcodeine in mobile phase. However, when the assay was repeated with samples in transformation media the resulting chromatograms revealed a variable large solvent front peak which partially and sometimes completely obliterated the morphine standard peak. It was not considered feasible to employ a standard that eluted after the codeine and norcodeine peaks. This was because the transformation levels normally encountered are of the order 5 to 20% norcodeine, which ensures that the codeine peak will be variable and about 5 - 10 times greater than the norcodeine peaks. As codeine elutes after the norcodeine, it would probably obliterate a standard if included. However, regression analysis results for peak heights and areas against concentration

of codeine and norcodeine in transformation media, indicated that the assay was unaffected by components in the media. This was further ratified by the excellent precision obtained for repeated injections of sample in transformation media (Table 2.13). It was concluded therefore that the Partisil 10 ODS-2 assay could be employed as an external standard procedure for the determination of codeine and norcodeine directly from transformation media.

A more sensitive assay was required for the determination of these drugs at the low transformation concentrations that may be encountered in enzyme kinetic studies. Column C (Table 2.1) packed with ODS-Hypersil 5 μm was employed in an attempt to achieve this, and the systematic procedure established in the previous section to select a mobile phase for Column B was now repeated for Column C. The more compact nature and smaller particle size of ODS-Hypersil, compared with Partisil 10 ODS-2, created a higher back pressure in the system, which restricted the mobile phase flow rate. This was overcome by replacing methanol with a less viscous organic modifier, acetonitrile, in the mobile phase. Acetate buffer was replaced with a phosphate buffer to improve the column efficiency and reduce peak tailing, but the most significant modification of all, was the addition of an ion-pairing agent to the mobile phase. Pentane sulphonic acid was employed, and the pH adjusted so that both codeine and norcodeine paired with it, enhancing their retentions at the expense of the solvent front (Figure 2.6(A)).

The results of the HPLC assay of codeine and norcodeine, using the ODS-Hypersil column, demonstrated a tenfold increase in sensitivity over the Partisil 10 ODS-2 method with detection limits of 1×10^{-4} mg/ml, and a linear range tested of 1×10^{-4} mg/ml to 25×10^{-2} mg/ml. Furthermore, there was no interference from the solvent front, illustrated by a typical chromatogram of the codeine-norcodeine separation in transformation media (Figure 2.6(B)), and the precision was unaffected when repeated in transformation media (Table 2.15). It was concluded that the ODS-Hypersil 5 μm HPLC assay was suitable for the direct analysis of codeine and norcodeine in transformation media, and sensitive enough for enzyme kinetic studies.

2.8.2 Isolation and purification methods

Hydrophobic surfaces of non polar C_8 or C_{18} reversed-phase HPLC materials have been employed previously for the extraction of a wide range of organic molecules[32]. Little and Fallick[89] reported on the use of very large injection volumes of up to 200 ml for relatively non polar compounds applied from an aqueous solution to a C_8 reversed-phase column. Similar techniques were reported for peptides and alkaloids injected from aqueous solutions[90,91]. The relatively non polar organic species are injected from an aqueous solution onto the hydrophobic surface, and become immobilised until the elution strength of the solvent mixture is increased. This allows the compound(s) to concentrate into a very small zone on top of the reversed-phase column. The components are eluted with a

suitable agent usually with little band broadening, and this provides a method for the extraction of components from biological samples such as transformation mixtures.

The extraction of codeine and norcodeine from transformation media by the Partisil 10 ODS-2 reversed-phase material was found to depend on the pH of the transformation media, the addition of methanol to the transformation media before extraction, transformation media flow rate through the column, the polarity of the eluting solvent and the temperature at which the extraction was achieved (Table 2.17). Optimum extraction efficiencies for codeine and norcodeine of 70% and 68% respectively (and 6.86 and 6.66 mg g^{-1} test compound per gram of packing material respectively). were obtained at a pH of 8.5, with 5% v/v added methanol, at a flow rate of 5 ml/min, with acetone as the eluting solvent and at a temperature of 4^{o}C. However, it was considered that the results could be improved if a more suitable material was employed.

The extraction of drug molecules from body fluids with Amberlite resins has been shown to be comparable, if not better than, conventional liquid/liquid extraction techniques in terms of time, efficiency and cost[92-94]. For example, procedures for the rapid, efficient and reproducible extraction of morphine[92], amphetamine and phenobarbitone[93] from urine by XAD-2 resins have been described with recoveries of 90 - 100% The resin extraction procedure involves single adsorption and desorption steps, and the overall resin-extraction efficiency is dependent

upon several parameters including the pH of the media, flow rate of media through the column, polarity of the eluting solvent, and the flow rate of the eluent[93].

Of the resins examined (Table 2.18) the best recoveries of codeine and norcodeine from transformation media were achieved with XAD-4 resin. This occurred at a pH of 9.5, flow rates of 1 ml/min for media and the eluent through the column, and using acetone to elute the components from the column. Under these conditions 95% norcodeine and 76% codeine were extracted from the media, with values of 12.6 mg g^{-1} and 9.9 mg g^{-1} respectively for these compounds per gram of XAD-4 resin. This represented a considerable improvement over the Partisil 10 ODS-2 material.

The parameters which affect the extraction of drugs from biological fluids by Amberlite resins have been discussed by Miller *et al.*[92]. It is possible that the same parameters apply to drug extractions with reversed phase HPLC materials. These authors found that the extraction of morphine from urine by XAD-2 resin was significantly influenced by the urine pH. Adsorption of morphine by the non-ionic resin was thought to occur by Van der Waals forces[92], hence it was highest at the pH where morphine is electrically neutral, and adsorption was decreased at pH's where the phenol or amine function of the morphine molecule were ionized. The extraction of codeine and norcodeine from transformation media, with both Partisil 10 ODS-2 reversed phase HPLC material, and the Amberlite resins, increased with the pH of the media (Tables 2.17 and 2.18). Incorporating

the pKa values for codeine and norcodeine (determined in Section 2.4.1) into Equation 2.7 shows that only 0.25% codeine and 1.96% norcodeine is ionized at pH 10.5. This could explain the higher extraction efficiencies at alkaline pH's, because the non-ionized drug will be less soluble in aqueous media, and more likely to be adsorbed onto the non-ionic resin surface. With the reversed phase procedure the addition of 5% v/v methanol to the transformation media was found to improve the extraction results (Table 2.17). The additional methanol may serve to increase the reversed phase to water surface area by keeping the brush surface wetted and open during the adsorption stage of the extraction procedure. Another parameter influencing the adsorption of codeine and norcodeine from the transformation media was the flow rate of media through the column (Table 2.17). Miller *et al.*[92], comparing the extraction efficiency of morphine from urine when passed through an XAD-2 resin column at gravitational flow rates and at controlled flow rates found that increased adsorption efficiency and reproducibility were obtained when the flow was controlled, and generally the adsorption was improved at lower flow rates. A controlled flow rate of 1 ml min^{-1} was subsequently employed for the extraction of codeine and norcodeine from transformation media using the Amberlite resins.

The desorption of drugs from the resin is a competition between the resin adsorption forces and the ability of an organic solvent to solubilise the drugs adsorbed onto the resin surface[92]. Thus acetone was found to be more efficient than 1,2-dichloroethane,

or 1,2-dichloroethane (80); propan-2-ol (20) for the elution of codeine and norcodeine. The addition of polar propan-2-ol would be expected to be beneficial only if the drugs used were significantly polar.

Finally, the experimental temperature at which the extraction was conducted influenced the results (Table 2.17). The extraction efficiency was improved at the lower temperature of $4^{o}C$. However, it was not considered practicable or economical to use such a temperature on a continuous basis.

As a result of the group extraction experiments, XAD-4 resin was selected for the extraction of codeine and norcodeine from transformation media,and using a 100g of XAD - 4 resin these drugs were simultaneously recovered from media (250 ml). with extraction efficiencies of 85.8% and 98.5% respectively.

There are two parameters generally used to describe the performance of a preparative HPLC system[95]. These are the 'loading capacity' defined as the maximum amount of sample that can be applied without impairing the separation efficiency, and the 'throughput' defined as the amount of pure substance that can be separated per unit time. The design and operation of a preparative system is usually based on optimising these parameters with respect to time, solvent consumption and the smallest possible dilution of the sample in the effluent. Theoretical and experimental investigations have been carried

out to find rules for the selection of the optimal working conditions in preparative HPLC[96]. They have shown that the loading capacity and throughput are determined by a series of parameters, of which the most important are column length and diameter,linear flow velocity, particle size, sample size and concentration, number of theoretical plates required and separation efficiency of the column in terms of plate height and selectivity[96]. It has also been demonstrated[97] that as soon as the phase system, column dimensions and particle size have been selected the throughput is proportional to $(v/h)^{\frac{1}{2}}$ where v is the reduced velocity and h is the reduced plate height. This means that one must work under conditons where v/h is as high as possible, and h is as low as possible, for a particular mobile phase flow rate.

Adopting these principles, the optimum mobile phase flow rate for the codeine-norcodeine separation on the LiChroprep Si 60 column (D, Table 2.16,) was determined from plots of h against v, and $(v/h)^{\frac{1}{2}}$ against v, (Figures 2.8(a), (b)) to be 25 ml min^{-1}. Column loading studies performed at the optimum flow rate revealed a loading capacity for the complete separation of equal quantities of codeine and norcodeine on Column D to be 3.76 mg codeine and norcodeine per gram of LiChroprep Si 60 silica.

The continuous injection of large amounts of sample and/or dirty samples (such as transformation media) can contaminate any HPLC column.Strongly adsorbed contaminant may accumulate

gradually on the packing material. If they are not washed off the column, and for example, the eluting power of the solvents used remains constant, then the effect can be seen on decreased kappa (κ) values for the compounds tested and a reduced resolution. HPLC columns are expensive[97]. and therefore it is desirable to salvage existing columns by some method of regeneration without unpacking and repacking them. The final theoretical plate count for the regenerated column should be close to the original one, and the surface properties and activity of the final column should be similar to the original. A thermal method of regenerating column D was attempted (Section 2.7.5) and the final column was checked for activity by repeating the column loading studies. However, the retention parameters, kappa (κ), column efficiency ($N_{0.1}$), resolution (Rs) and the symmetry factor, plotted against column loading of codeine and norcodeine, before and after column regeneration (Figures 2.9 to 2.12) show that there was only a marginal improvement in column performance using this method of regeneration. Subsequently, Column D was repacked with fresh LiChroprep Si 60 silica, and the column loading studies repeated once more (see Figures 2.9 to 2.12 for the column performance results). As a consequence it was found that the column would separate 135 mg each of codeine and norcodeine in a sample, with 100% purity, equivalent to a loading capacity of 5.12 mg g^{-1} These values were significantly greater than those obtained with the original and regenerated columns respectively with LiChroprep Si 60. However, it was thought that the employment of a reversed phase material may

improve the loading capacity further, with the advantage that it might be used for longer periods of time without the need for column regeneration. Hence, further attempts were made to improve the loading capacity by utilising LiChroprep RP18, 25 - 40 μm material with a 22% carbon loading, selected for its high retention and loading characteristics. The loading capacity obtained for the complete separation of codeine and norcodeine (98% purity) was 9.48 mg g^{-1} and equivalent to 552mg codeine base and 450 mg norcodeine base in a 10 ml volume injection. The limiting factor was not the capacity of the column to separate the codeine from the norcodeine, but the solubility of these compounds in mobile phase. It was not considered feasible to employ a larger volume loop in this case because of the problems associated with injecting the sample with the column, For example, it may be necessary to use a second pump to do this.

A scheme was designed to demonstrate the efficiency of the combined extraction procedures developed to recover codeine and norcodeine from transformation media (Figure 2.15). A 7 litre batch of fermentation liquor from a fermentation run was processed, and the % recovery after each stage of the extraction scheme determined by analytical HPLC(Table 2.21) The complete beatch of liquor was initially passed through the 100 g XAD-4 resin column to extract the drugs on a group basis. This was achieved by dividing the sample into three equal portions because the total quantity of drugs in the whole sample exceeded the extraction capacity of the resin. The recovered sample was then divided into two equal portions, and one sample

was passed through the straight phase preparative column (D) and the other portion through the reversed phase preparative column (F), to isolate pure codeine from norcodeine. An extra extraction step was required after the reversed phase separation to recover the drugs from the aqueous mobile phase. Consequently, the final recovery of the drugs from the fermented sample (Table 2.21) employing the reversed phase proecedure was slightly lower than the recovery employing the straight phase procedure (71.25% codeine and 80.69% norcodeine, compared to 79.20% codeine and 83.97% norcodeine respectively). However, the reversed phase system may be more economical in long term use compared to the straight phase system. The mobile phase employed for the reversed phase system was simpler (water combined with an organic modifier) and hence less expensive,as opposed to the tertiary component organic mobile phase employed for the straight phase system. In addition the reversed phase column was found in practice to provide longer service without the need for regeneration or repacking. Finally, the throughput and loading capacity that could be achieved with the reversed phase column for codeine and norcodeine was found to be far superior to the straight phase column material. This is most important in batch work to cope with the sample load, using the least solvent and analysis time.

CHAPTER THREE

THE N-DEMETHYLATION OF CODEINE BY SUBMERGED CULTURES OF *Cunninghamella* Spp.

Chapter 3. The N-demethylation of codeine by submerged cultures of *Cunninghamella* sp.

3.1 Introduction

The growth and transformation ability of submerged cultures of *Cunninghamella* species have been demonstrated in previous studies by Sewell[18]. A codeine substrate was N-demethylated with high reproducibility when a two-stage growth and incubation protocol was adopted. Employing glucose as the carbon source, codeine N-demethylation was only observed after all the glucose was depleted from the growth medium. This finding was attributed to glucose inhibiting the synthesis of the N-demethylase enzyme in a form of catabolic repression[18]. This phenomenon was not observed when succinate was employed as the carbon source.

Several workers have demonstrated the ability of *Cunninghamella* sp. to utilise n-alkanes for the construction of biomass[98-100]. More recent studies have shown the possible involvement of monooxygenases in the metabolism of gaseous alkanes and hydrocarbons[101]. The metabolism of ethane, propane and butane by the fungus *Acremonium* required the presence of NADPH, and was inhibited by carbon monoxide, but not cyanide, characteristic of monooxygenase activity[101]. More conclusive evidence for the association of n-alkanes with monooxygenase activity has been demonstrated in yeasts[102]. Lebeault *et al.*[102], for example, reported the presence of a cytochrome P-450 type monooxygenase in *Candida tropicalis* utilising tetradecane as the sole carbon source. This system was capable of effecting the

ω-hydroxylation of fatty acids and alkanes, showed a dependence on molecular oxygen and NADPH, and was inhibited by carbon monoxide, but not cyanide. The hydroxylase activity was induced by the presence of tetradecane dispersed in the medium, but not by the presence of glucose[102]. Similar studies have shown that other suspected monooxygenase enzymes can be induced by growing cultures on hydrocarbon sources and in particular, tetradecane[103-104].

The aim of this study was to compare the growth and transformation ability of submerged cultures of *Cunninghamella* in the presence of tetradecane; succinate and glucose carbon sources. Examination of the transformation profiles would show if tetradecane is capable of inducing the N-demethylase enzyme in *Cunninghamella* resulting in an increase in codeine N-demethylation.

3.2 Materials

3.2.1 Microorganisms

C. echinulata: IMI 199844; obtained from the Commonwealth Mycological Institute, Kew, Surrey

C. bainieri: C43; obtained from the American Cyanamid Company, Lederle Laboratories, Pearl River, New York, U.S.A.

Microorganisms stored under liquid nitrogen were transferred to Malt Extract Agar Slopes and incubated for 2 - 5 days at 27°C. The stock slopes were maintained at 4°C and subcultured at intervals not exceeding 3 weeks.

3.2.2 Growth media

a) Stock slopes:

Malt Extract Agar (Oxoid Ltd.) was prepared according to the manufacturer's directions. 10 ml.volumes were added to 20 ml. screw-capped glass universal containers, sterilised by autoclaving at 121°C for 15 minutes, and then inclined during cooling to produce a slope.

b) Chemically defined basal media:

These were prepared from Analar grade reagents (BDH Ltd.), and glass distilled water:

Stage-one basal medium. (mg ℓ^{-1}); KH_2PO_4 (378.0), $Na_2HPO_4.12H_2O$ (989.0), $(NH_4)_2SO_4$ (667.0), $MgSO_4.7H_2O$ (28.0), $CaCl_2.2H_2O$ (5.6), $ZnSO_4.7H_2O$ (22.0), $MnSO_4.7H_2O$ (2.20), $CuSO_4.5H_2O$ (0.56), $FeSO_4.7H_2O$ (11.10), Na_2SO_4 (56.0), $NaMoO_4.2H_2O$ (5.60), disodium EDTA (66.70).

The above medium was prepared as a double strength solution, packed in 500 ml Schott bottles, and autoclaved at 121°C for 20 minutes.

Stage-two basal medium. Salts (mg ℓ^{-1}); K_2HPO_4 (3327.0), $NaH_2PO_4.2H_2O$ (732.0), $(NH_4)_2SO_4$ (500.0), $MgSO_4.7H_2O$ (290.0), disodium EDTA (290.0).

Trace elements (mg ℓ^{-1}); $CaCl_2.2H_2O$ (33.0), $(NH_4)_6Mo_7O_{24}.4H_2O$ (93.0), $FeSO_4.7H_2O$ (3.50), $ZnSO_4.7H_2O$ (5.50), $MnSO_4.4H_2O$ (0.75),

$CuSO_4.5H_2O$ (0.20), $Co(NO_3)_2.6H_2O$ (0.125), $Na_2B_4O_7.10H_2O$ (0.09). The salts mixture was prepared as a double strength solution and was sterilized by autoclaving at 121°C for 20 minutes. The trace elements were dissolved in distilled water to produce a 50 X concentrate and the solution was sterilized by membrane filtration (0.2μ pore size)

c) Amino acid mixture

A solution of 12 amino acids (Sigma London Chemical Co.) (mg ml^{-1}) was prepared in 10 ml. distilled water; Arginine (1.87), aspartic acid (4.69), glutamic acid (1.40), glycine (0.94), histidine (1.40), isoleucine (3.75), lysine (4.68), methionine (0.94), phenylalanine (1.87), threonine (1.87), tyrosine (0.94), valine (3.75). The mixture was sterilized by membrane filtration and used within 48 hours of preparation.

d) Carbon source solutions

Glucose and sodium succinate (Analar, BDH Ltd.) were dissolved in distilled water to produce 10% w/v solutions, and sterilized by membrane filtration. Tetradecane (Aldrich Chemical Co. Ltd.) was filter sterilized without dilution.

3.2.3 Chemicals and reagents

Tetradecane; Aldrich Chemical Co. Ltd.; n-hexane (HPLC grade); Fisons Ltd., Loughborough, U.K. Reagents used for the glucose determination were purchased from Sigma London Chemical Co. Ltd.:-

PGO enzyme capsules, each containing peroxidase from horseradish (100 Purpurogallin units), glucose oxidase from *Aspergillus niger* (500 I.U.) and buffer salts. The contents of one PGO capsule were dissolved in distilled water (100 ml) contained in a foil covered glass bottle to produce reagent A.

dianisidine dihydrochloride, preweighed vial (50 mg), the contents of which were reconstituted with distilled water (20 ml) to produce reagent B.

The combined enzyme-colour reagent (reagent C) was prepared by addition of reagent B (1.6 ml) to reagent A (100 ml). Reagent C was stored at $4^{o}C$ and used within 14 days of preparation.
Glucose standard solution, containing β-D-glucose (1.0 mg ml^{-1}) in benzoic acid solution (0.1%), which was stored at $4^{o}C$.

3.2.4 Equipment and instrumentation

Automatic pipettes: Triple range (0.2, 0.5 and 1.0 ml), Oxford Laboratories.
Variable range Gilson pipette; (Pipetman, 0.2 to 1.0 ml and 1 ml to 5.0 ml) Gilson Medical Electronics, France.
Pipette tips (polypropylene) were supplied by the respective pipette manufacturer. Tips were packed into DRG autoclave bags and sterilised by autoclaving at $121^{o}C$ for 20 minutes.
Culture flasks; 250 ml., borosilicate glass, corner baffled, and Kipps burettes (5 and 10 ml volumes); Gallenkamp Co. Ltd.
Filtration units; Swinnex units, Millipore Corporation, Massachusetts, U.S.A.

Rotary incubator; New Brunswick G25R, cooled, gyrotatory with 1" stroke, New Brunswick Scientific Co. Inc., New Jersey, U.S.A.
Gas Liquid Chromatograph; Packard Model 438, fitted with a flame-ionisation detector, Packard Instruments Ltd., Reading, England.
GLC column; 1 m, glass, 4 mm. i.d. packed under nitrogen with 3% SE-30 Ultraphase on Chromosorb W-HP, 100/120 mesh, Phase Separations, Queensferry, Clwyd.
GLC syringes; 1µl volume, Scientific Glass Engineering UK Ltd., London.

3.3 Methods

3.3.1 Conditions for the growth and incubation of cultures

Microorganisms were grown and incubated in submerged liquid cultures according to the 2 stage protocol described by Sewell[18]. Sterile growth medium for stage-one cultures consisting of:- stage-one basal medium (25 ml.), amino acid mixture (5 ml.), carbon source solution (5 ml) and distilled water (to 50 ml), was inoculated with mycelium from freshly prepared stock slopes of *Cunninghamella*. The stage-one cultures were incubated at 27°C with rotary agitation of 250 rpm for 3-4 days to produce a pelleted growth form. This was aseptically transferred to a sterile, 250 ml conical flask fitted with a sterile Kipps burette (10 ml). The flask was gently swirled to prevent settling of the mycelial pellets and 10 ml was dispensed into stage-two culture flasks containing sterile stage-two growth medium (stage-two basal medium

25.5 ml., amino acid mixture (5 ml.), carbon source solution (5 ml.) and distilled water to 50 ml.). Stage-two cultures were incubated at 27°C with agitation of 250 rpm for 24 hours. The codeine phosphate substrate was then added as a filter sterilised solution to give a final codeine concentration of 1 mM in each stage-two culture (unless otherwise stated), and incubation was continued, for the required period (10 days, unless otherwise stated).

3.3.2 Estimation of biomass

Growth of cultures was estimated by determination of the dry cell weight of the biomass.

Whatman 542 hardened, ashless filter papers, 9.0 cm diameter, were placed in petri dishes and dried in an oven at 70°C with the lids removed. The petri dish, filter and lid units were then allowed to cool to room temperature in a desiccator, and weighed on an analytical balance. The entire contents of the culture vessel were filtered through the filter paper in a Büchner apparatus under negative pressure. Vessels were rinsed with distilled water (2 x 10 ml.) which was also filtered. The filter papers plus retained material were transferred back to their respective petri dishes, dried in the oven at 70°C to constant weight, and allowed to cool to room temperature in a desiccator. The petri dish units were re-weighed to determine the dry cell weight (DCW) of the biomass.

3.3.3 Determination of glucose in transformation media

Glucose was determined by the glucose oxidase procedure of Raabo and Terkildsen[105]. The assay is based on the oxidation of a colourless indicator O-dianisidine by hydrogen peroxide formed from the oxidation of glucose. Oxidised dianisidine is coloured and is estimated spectrophotometrically.

a) Construction of the standard curve.

Standard solutions containing β-D-glucose over the concentration range 0.5 to 3.0 mg ml^{-1} in stage-two growth medium were prepared. Each standard solution was diluted 1 in 20 with distilled water and an aliquot (0.5 ml) was pipetted into a stoppered test tube. Reagent C (5.0 ml) was added to each tube and the contents mixed thoroughly. A blank was also prepared containing distilled water in place of the standard solution. All tubes were incubated in the dark at 37°C for 30 minutes. The tubes were then cooled to 20°C in a water bath and the absorbance of each mixture was measured spectrophotometically against the blank at 450 nm. A plot of the absorbance obtained against the concentration of the standard solutions is shown in Figure 3.1 and gave the following linear regression analysis;

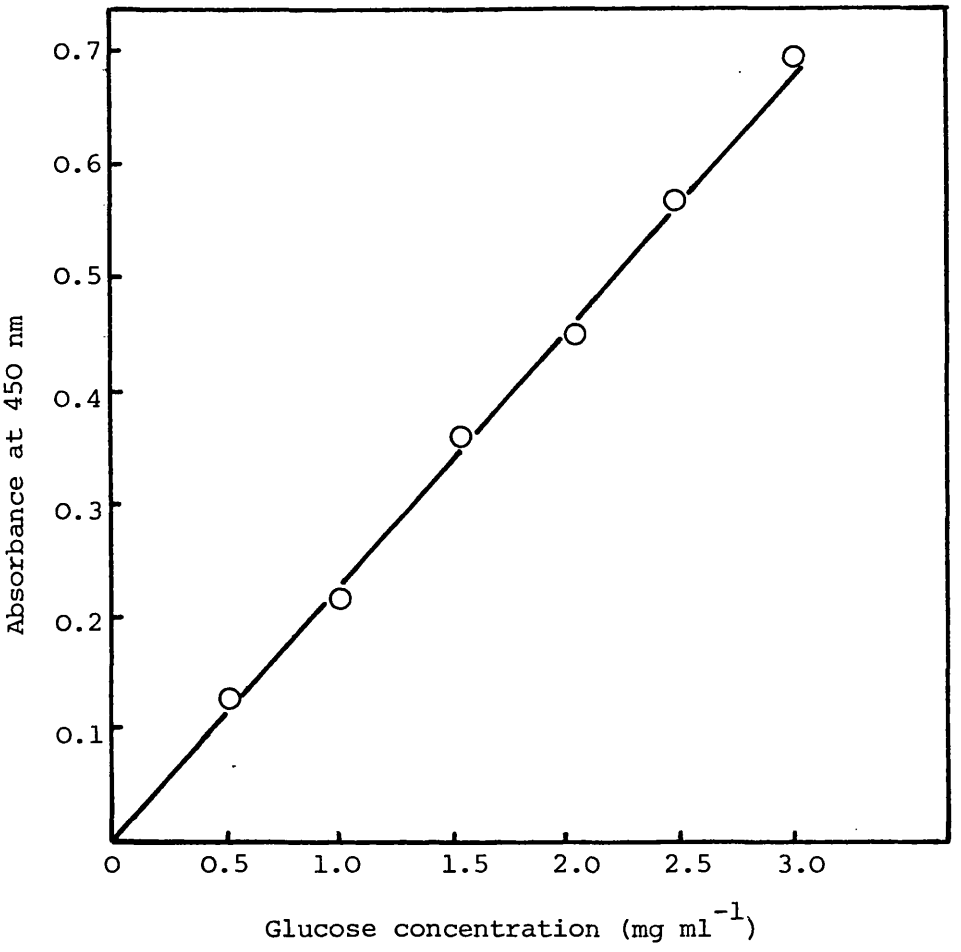
slope = 0.226

intercept = -0.0076

correlation coefficient = 0.998

The results show a direct correlation between the colour intensity produced in the assay and glucose concentration in the growth medium.

Figure 3.1. Standard curve of glucose in stage-two growth medium.



(b) Determination of glucose in transformation media

Transformation samples (5.0 ml) were centrifuged (3000 rpm, 15 minutes) to remove cellular material. Clear supernatant was diluted 1 in 100 with distilled water to give glucose concentrations within the standard curve. Aliquots (0.5 ml) of these solutions and also of a distilled water blank were mixed with reagent C (5.0 ml) and incubated at 37°C for 30 minutes. A 1 in 20 dilution of the standard glucose solution (1.0 mg ml^{-1}) was similarly treated. The absorbance of each solution (at 20°C) was read against the blank at 450 nm, and the glucose concentration was calculated from equation 3.1

$$\text{Glucose concentration (mg ml}^{-1}\text{)} = \frac{\text{Absorbance 450 nm of test} \times 5}{\text{Absorbance 450 nm standard}} \quad \ldots 3.1$$

3.3.4 Determination of tetradecane in the transformation media

Tetradecane in the transformation media was determined by the GLC procedure described below.

(a) Assay conditions; A glass column (1 m x 4 mm i.d.) packed with SE-30 stationary phase and nitrogen carrier gas, at a flow rate of 15 ml. min^{-1}, were employed. The flow rates of hydrogen and air to the flame ionization detector were 25 and 250 $ml.min^{-1}$ respectively. With the oven temperature and injection temperature both at 190°C, and the detector temperature at 210°C, the retention time for tetradecane was 4 minutes.

(b) Selection of internal standard; A known amount of an internal standard was added to the sample to compensate for minor fluctuations in the sample volume injected. The ratio of peak height of the analyte peak divided by peak height of the standard peak, is independent of the sample volume injected, provided that the amount of each component injected is within the linear region of the assay. Tetradecanol was selected as the internal standard and had a retention time of 10.6 minutes for the assay conditions described in 3.3.4(a). A typical chromatogram is shown in Figure 3.2 with a summary of the assay conditions.

(c) Calibration procedure:

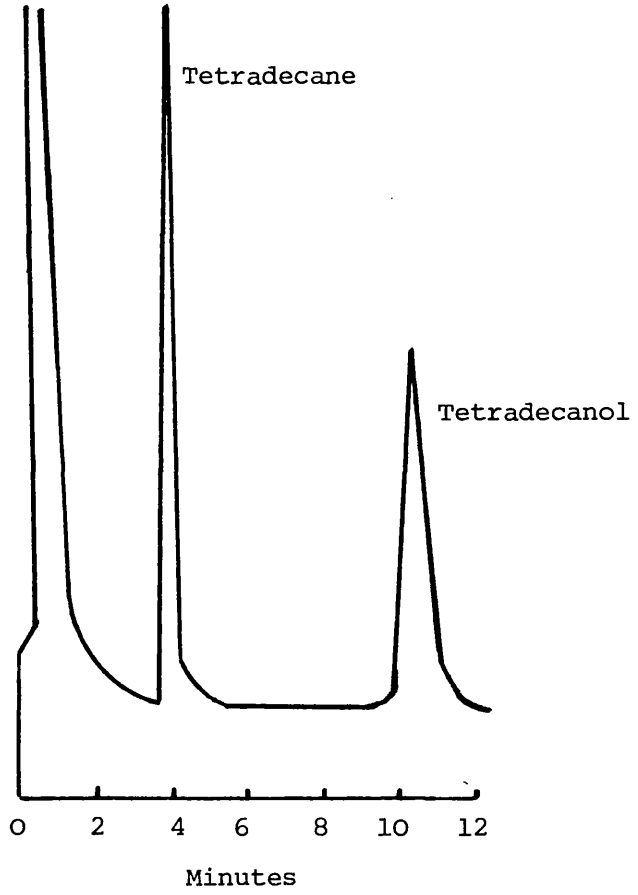
A series of calibration solutions was prepared by adding 4 ml of a solution of tetradecanol in hexane (5 μg ml^{-1}) to the sample volumes of tetradecane shown in Table 3.1 contained in glass tubes and adding hexane to give a final volume of 5 ml in each case. The tubes were capped and the samples mixed on a vortex mixer for 2 minutes. Each sample was assayed in duplicate by the GLC procedure injecting 1 μl volume each time. The continuous line in Figure 3.3 shows a plot of the peak height ratio of tetradecane/ tetradecanol against the concentration of tetradecane in hexane in the calibration samples, and gave the following regression analysis

slope = 5.075

intercept = -0.0014

correlation coefficient = 0.9998

Figure 3.2. Example of GLC chromatogram obtained in the assay of tetradecane



Analytical conditions

Column; 3% SE-30 on Chromosorb W-HP, 1 m x 4 mm i.d.

Carrier gas; nitrogen, flow rate 15 ml min^{-1}

Oven temperature; 190°C

Detection; F.I.D.

Injection volume; 1 µl.

Table 3.1. Preparation of samples for the GLC calibration of tetradecane

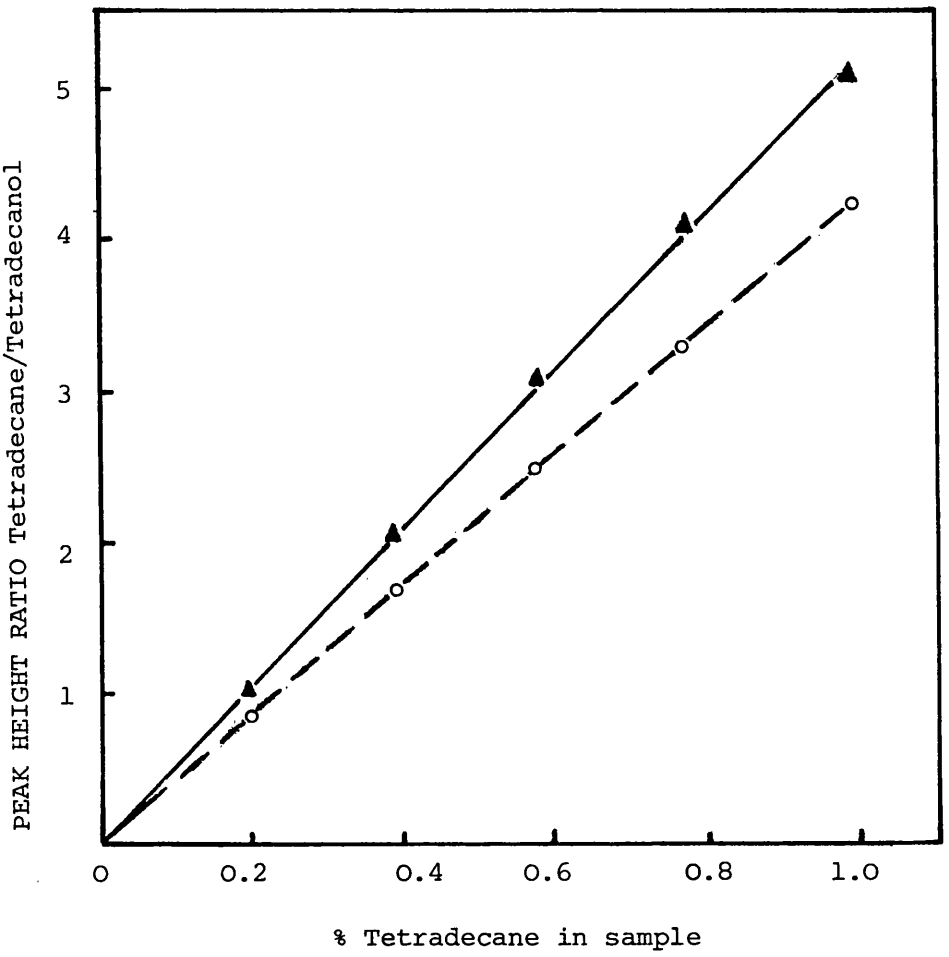
Tetradecane sample volume (µl)	Equivalent concentration in 5 mls % v/v
10	0.2
20	0.4
30	0.6
40	0.8
50	1.0

The results indicated that the GLC assay was suitable for the estimation of tetradecane in hexane. It was therefore used to determine the extraction efficiency for the extraction of tetradecane from transformation media.

(d) Extraction of tetradecane from transformation media; Sample volumes ranging from 100 µl to 500 µl of tetradecane were transferred to test tubes (100 ml) each containing transformation medium (50 ml.). The tubes were capped and each mixture was vortexed for 2 minutes to disperse the tetradecane. A sample of the homogeneous mixture (2 ml.) was pipetted into a glass vial containing 0.5% tetradecanol in hexane (2 ml) and the mixture vortexed again for 2 minutes. Samples (1 µl) of the hexane upper layer were analysed by the GLC assay.

The broken line in Figure 3.3 shows a plot of the peak height

Figure 3.3. Data for the extraction and GLC analysis of tetradecane from hexane (▲) and transformation medium (○).



ratio of tetradecane to internal standard against the concentration of tetradecane in the growth medium samples. The regression analysis was as follows:

slope = 4.175

intercept = -0.007

correlation coefficient = 0.999

and showed that the combined GLC and extraction procedure was suitable for the determination of tetradecane in transformation medium. The extraction efficiency was determined from equation 3.2

$$\text{Extraction efficiency} = \frac{\text{slope of recovery plot from media}}{\text{slope of recovery plot from hexane}} \times 100\% \quad \ldots 3.2$$

and was 89.8% for the extraction of tetradecane from transformation medium into hexane.

3.4 Experimental

3.4.1 The effect of carbon source on codeine transformation

C. bainieri (C43) was cultured by the two-stage procedure described in Section 3.3.1 (page 133). The carbon sources investigated were glucose, succinate and tetradecane. These were added to both stage-one, and stage-two growth media, to provide an initial concentration of 1% w/v. The test substrate codeine phosphate (1 mM) was added to stage-two cultures after 24 hours incubation at 27°C, 250 rpm. Sufficient stage-two cultures were prepared to enable samples to be taken at 2 - 3 day intervals during

the course of the transformation for the determination of growth, carbon source depletion and codeine N-demethylation.

On completion of the incubation period the growth was determined by the dry cell weight procedure described in Section 3.3.2. Samples of the filtrate retained from the dry cell weight determinations were assayed for glucose by the procedure described in Section 3.3.3. In the case of cultures grown on a tetradecane carbon source, samples were assayed for tetradecane employing the procedure described in Section 3.3.4. Codeine transformation was determined in all cultures by assaying the filtrate for norcodeine by the Partisil 10 ODS-2 reversed phase procedure described in Section 2.4.4 and the external standard method described in Section 2.3.3(b).

The total dry cell weight (mg) obtained for a transformation culture was divided by the final volume to give the dry cell weight in units of $mg.ml^{-1}$. Codeine transformation was expressed from the norcodeine concentration in transformation mixtures and calculated from equation 3.3.

$$\text{Norcodeine}(\mu M) = \frac{\text{Amount norcodeine } (\mu M.)}{\text{Amount codeine + norcodeine } (mM)} \quad \ldots 3.3$$

In addition, the specific transformation was calculated for each culture flask from equation 3.4:

$$\text{Specific transformation} = \frac{\text{Norcodeine concentration in the transformation mixture } (\mu M)}{\text{Total dry cell weight of culture}(mg)} \quad \ldots 3.4$$

to give a measure of the transformation of codeine obtained per unit DCW independent of the biomass at a given incubation time.

Progress curves for microbial growth (dry cell weight) and specific transformation obtained with the carbon sources glucose, succinate and tetradecane in the growth media, are shown in Figure 3.4. Progress curves for the depletion of glucose and tetradecane, and for the transformation of codeine are shown in Figure 3.5.

It can be seen from Figure 3.4 that *C. bainieri* did not grow as well in the presence of succinate or tetradecane carbon sources as it did in the presence of glucose. However, the specific transformations obtained in the presence of succinate and tetradecane were both significantly higher than those obtained with glucose.

The profiles shown in Figures 3.5 show that the glucose and tetradecane carbon sources were utilised during the course of the incubation period. The transformation of codeine in the presence of glucose (Figure 3.5) demonstrated a definite lag phase, whereas this was not evident in the presence of tetradecane. The significance of these findings are discussed on page 152.

3.4.2 The effect of substrate concentration on codeine transformation

In Section 3.4.1 the initial concentration of codeine phosphate test substrate in the stage-two culture media was 1 mM. Previous studies by Sewell[18] showed that this substrate concentration

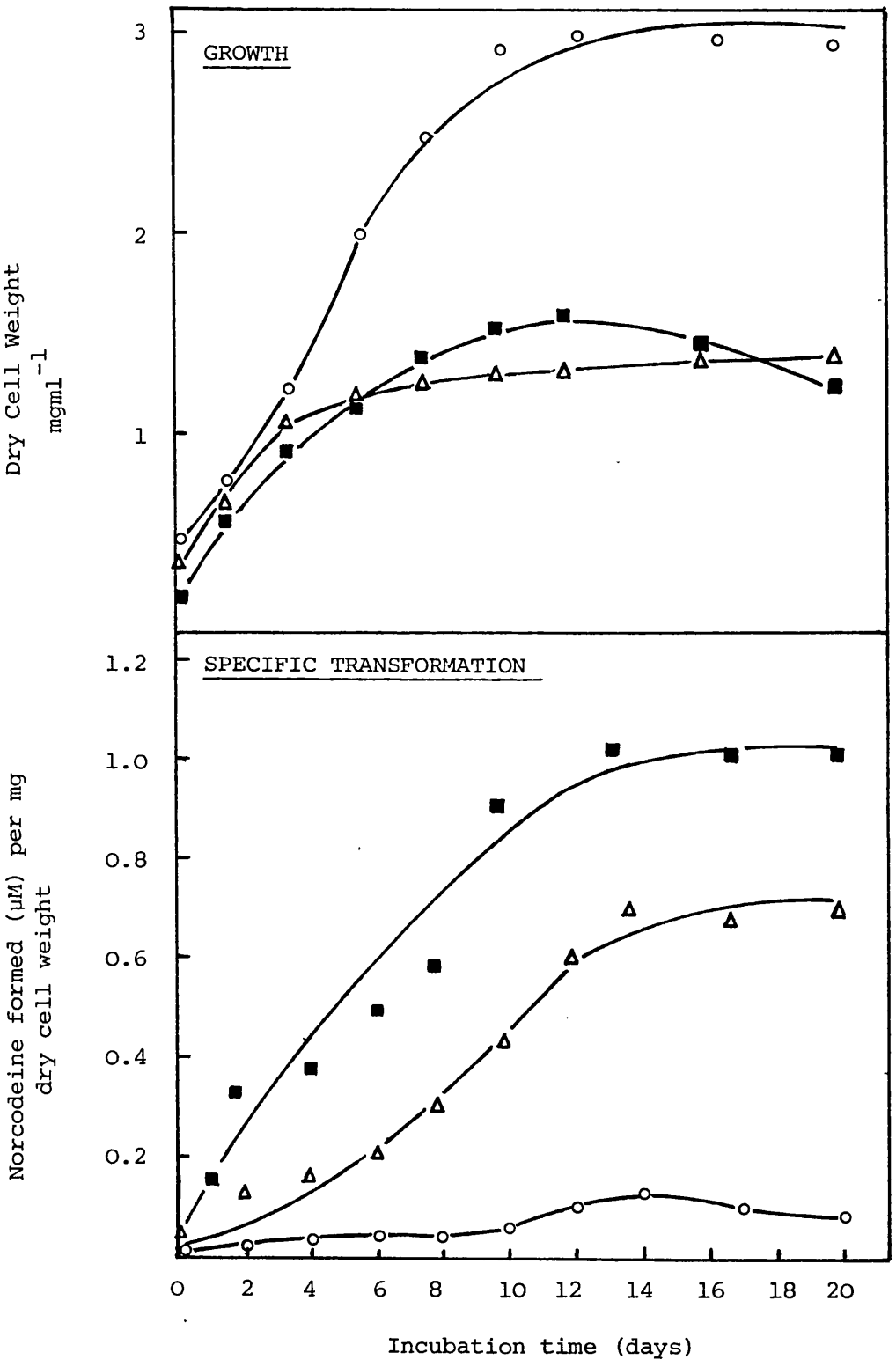


Fig. 3.4. Microbial growth and specific codeine transformation by *C. bainieri* incubated at 27°C, 250 rpm with carbon sources, glucose O , succinate Δ and tetradecane ■.

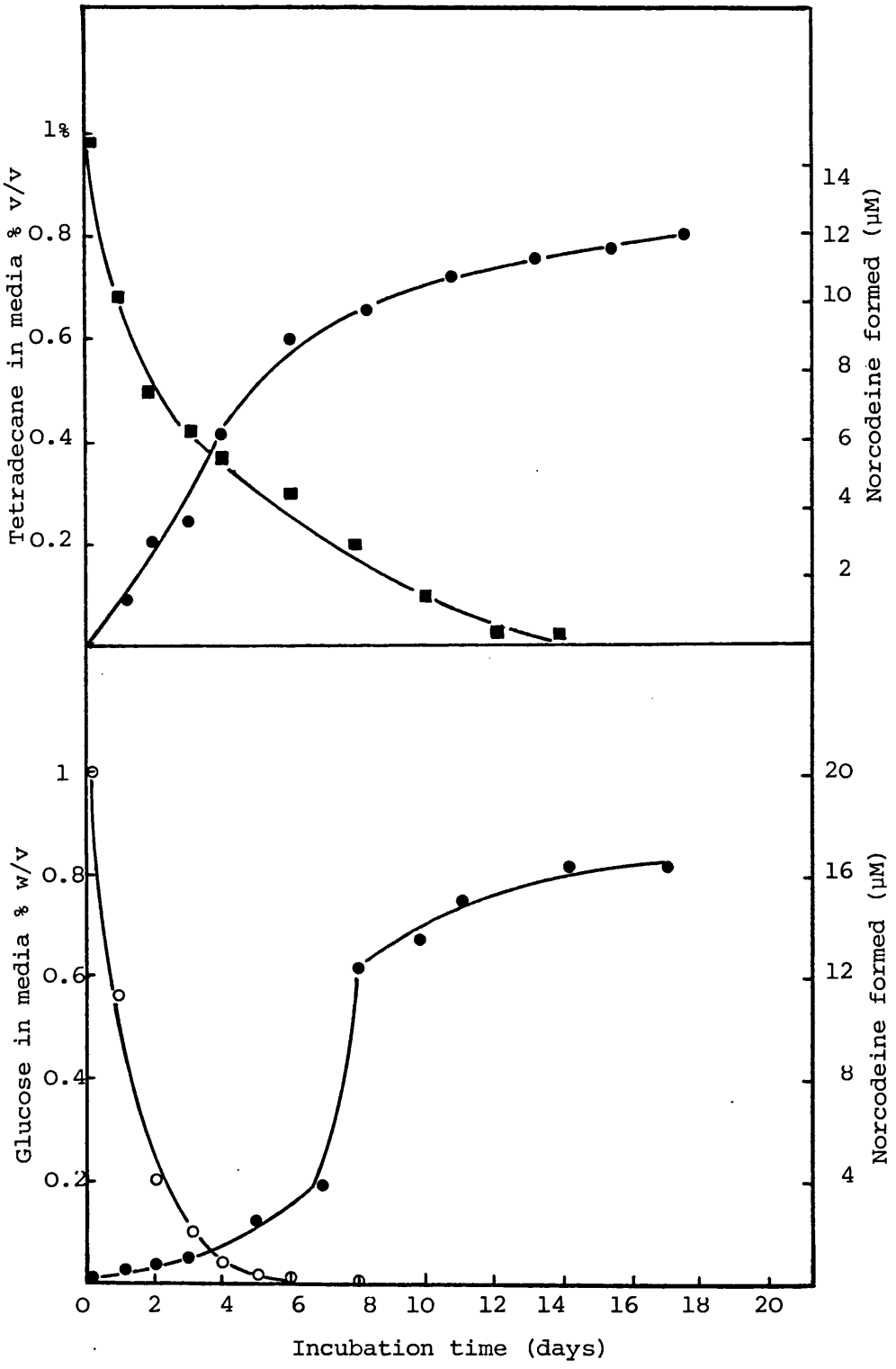


Figure 3.5. Tetradecane concentration ■ and glucose concentration o in the growth medium and codeine transformation ● by *C. bainieri* incubated at 27°C, 250 rpm, in stage-two growth media.

resulted in the maximum transformation of codeine by *C. echinulata* when grown and incubated under similar conditions with a glucose carbon source. Above 1 mM the codeine transformation fell dramatically, a phenomenon which was attributed to substrate inhibition by codeine phosphate[18]. The results from Section 3.4.1 showed that cultures of *C. bainieri* grown on 1% tetradecane or succinate produced significantly higher specific codeine transformations than cultures grown on glucose. The aim of this study was to find out if cultures of *Cunninghamella* grown on succinate and tetradecane could tolerate higher codeine concentrations in the media and give higher codeine transformations than cultures grown on glucose.

Stage-two cultures of *C. bainieri* and *C. echinulata* were each prepared in stage-two growth medium (50 ml) containing one of each of the carbon sources (1%) glucose, succinate or tetradecane. Each culture was inoculated with the respective stage-one growth (10 ml) cultured in the same media. All cultures were incubated at 27^{o}C, 250 rpm, for 24 hours, and codeine phosphate was added to each culture as a filter sterilised solution. A range of codeine concentrations between 0.02 mM and 8.0 mM was examined. The stage-two cultures were incubated for a further 10 days under the same conditions and samples of each were assayed for the determination of growth (dry cell weight) and codeine N-demethylation (norcodeine formed μM and specific transformation).

The microbial growth and codeine transformation obtained with different substrate concentrations for *C. bainieri* and *C.*

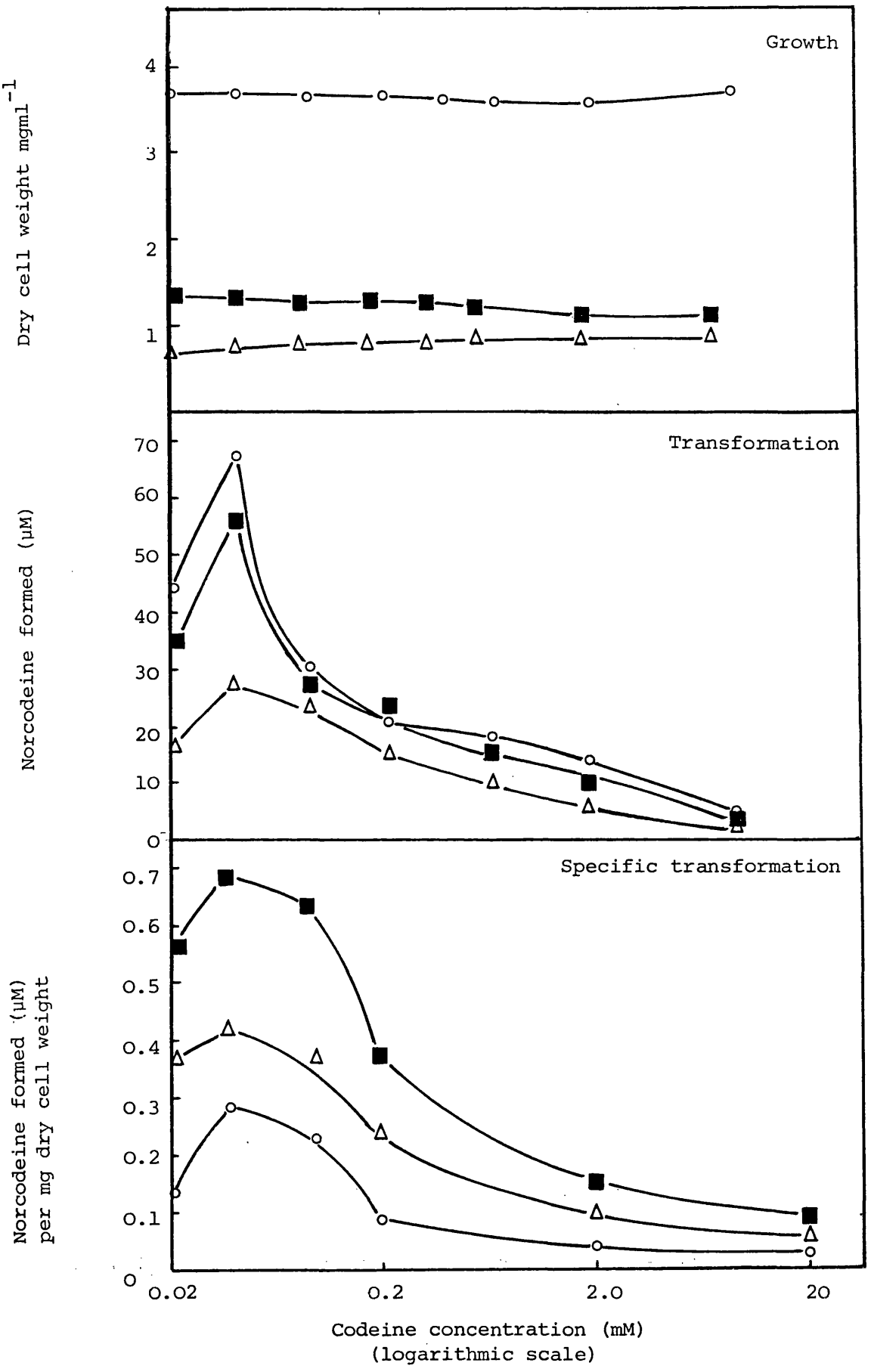


Figure 3.6. Microbial growth, codeine transformation and specific transformation by *C. bainieri* incubated in the stage-two growth medium with glucose (1%) o , succinate (1%) Δ and tetradecane (1%)■ Carbon sources at various substrate concentrations.

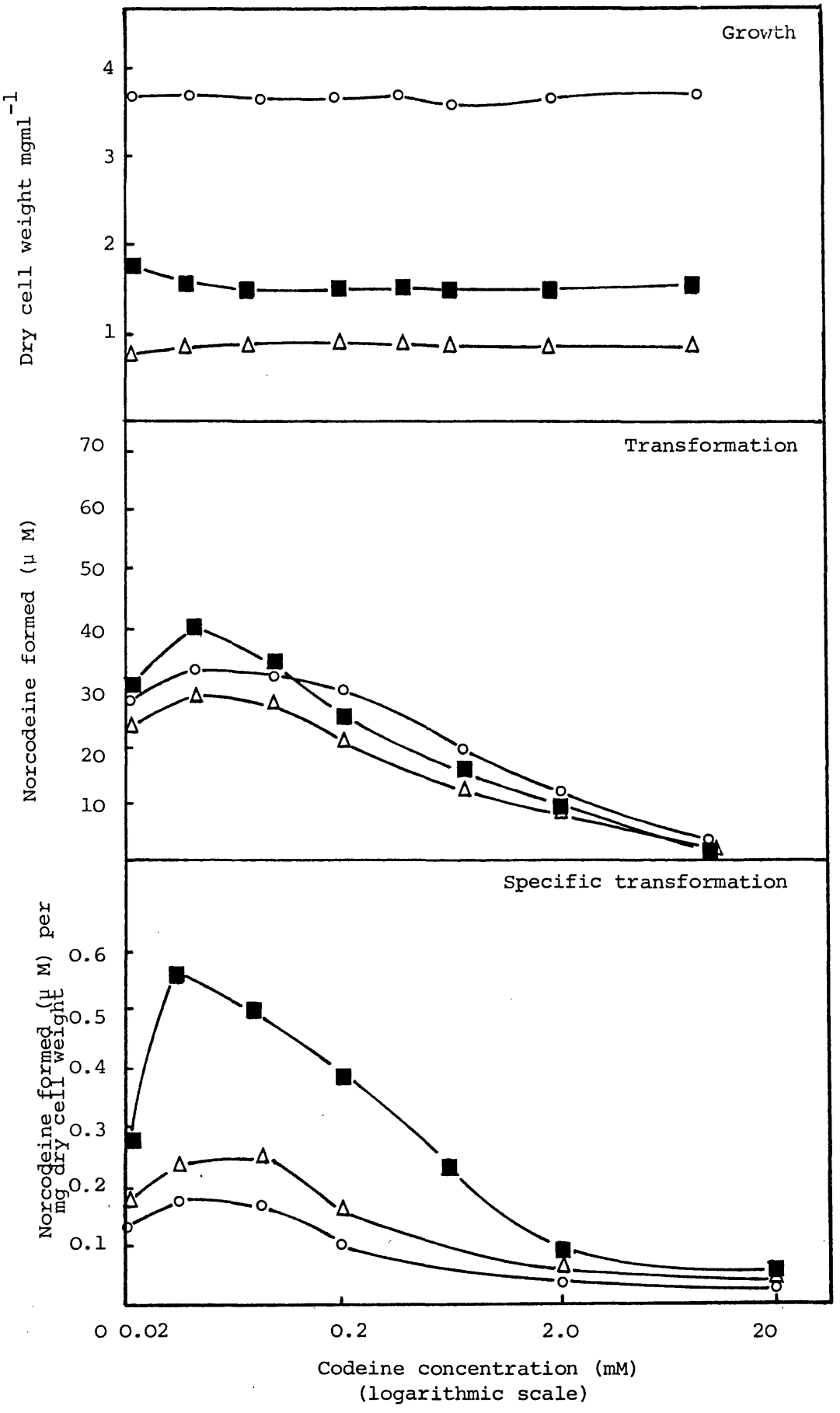


Figure 3.7 Microbial growth, codeine transformation and specific transformation by *C. echinulata* incubated in the stage-two growth medium with glucose (1%) o , succinate (1%) Δ and tetradecane (1%) ■ carbon sources at various substrate concentrations.

echinulata grown in the presence of glucose, succinate and tetradecane are shown in Figures 3.6 and 3.7.

The maximum codeine transformations and specific codeine transformations by all the cultures studied, and with all carbon sources studied, occurred at a substrate concentration of approximately 0.04 to 0.08 mM. Cultures grown on a tetradecane carbon source exhibited higher specific transformations than those grown on succinate or glucose, but the actual yield of norcodeine was similar for cultures grown on all the carbon sources investigated.

3.5 Discussion

The dry cell weight data in Figure 3.4 demonstrate the ability of *C. bainieri* to grow satisfactorily in the chemically defined growth media containing glucose, succinate or tetradecane carbon sources. However, the maximal biomass production after 20 days incubation was obtained with cultures grown on glucose. The limited growth obtained with succinate may be attributed to an unfavourable culture pH of 7.9 after two to three days incubation. Further examination of Figure 3.4 shows that the initial rate of growth on all carbon sources investigated was similar over the first 6 - 8 days of incubation. After this period only cultures on glucose continued to grow at the same initial rate. Studies by Hoffmann and Rehm[99] with *C. echinulata* grown on long-chained n-alkanes, including tetradecane, showed that these carbon sources were metabolised in a terminal degradation pathway to produce primary

alcohols and aldehydes, which were in turn converted to fatty acids. The resulting acids were then metabolised by β-oxidation or inserted directly, or after elongation with C_2 units, into the fungal lipids of the cell wall. Furthermore, they found[99] that after 8 days incubation *C. echinulata* produced higher yields of biomass on n-alkanes compared to glucose, but after 30 days incubation the reverse was true. The reasons for these observations were that in the exponential phase *C. echinulata* incorporated the degradation products of n-alkane metabolism directly into the lipids of the cell wall resulting in an increase in biomass. However, in the stationary phase the lipids were used to a considerable extent for cellular metabolism, as all the carbon source was depleted from the growth medium[99]. This may explain the growth curve shown for *C. bainieri* in Figure 3.4 on tetradecane carbon source.

Despite their limited growth, the cultures grown in the presence of succinateand tetradecane demonstrated higher specific transformation activities than cultures grown on glucose (Figure 3.4). In addition, comparison of the transformation profiles obtained for cultures grown in glucose and tetradecane (Figure 3.5) shows different patterns. The glucose cultures were the most productive, in terms of norcodeine yield, when the carbon source in the culture medium was significantly depleted (after day 5 or 6). However, the tetradecane cultures demonstrated codeine transformation throughout the microbial growth phase, despite the fact that the tetradecane was not depleted until day 11 to 12 of incubation. This glucose effect was also observed by Sewell[18] for the N-demethylation of

codeine by cultures of *C. echinulata*, and was thought to be caused by the catabolic repression of the N-demethylase enzyme by the readily utilised glucose carbon source. Once the glucose was depleted from the medium the enzyme was no longer inhibited, and transformation increased. As both succinate and tetradecane are relatively poorly utilised carbon sources this may explain why no apparent enzyme repression occurred in their presence. Since the cultures grown on tetradecane demonstrated higher codeine transformations than those on glucose or succinate, it may be concluded that tetradecane was a good inducer of the N-demethylase in *C. bainieri*. Further experiments were performed in Section 3.4.2 to find if tetradecane cultures would produce higher codeine transformations if the substrate concentration was increased. The results with cultures of *C. bainieri* and *C. echinulata* are shown in Figures 3.6 and 3.7 respectively. As the codeine phosphate substrate concentration in the transformation mixture was increased, the biomass of all the cultures investigated, and for all carbon sources investigated, remained reasonably constant. This indicated that growth of the cultures was not affected by the presence of codeine in the concentration range investigated. The highest growth was obtained with carbon sources in the order glucose > tetradecane > succinate.

There was a significant decrease in transformation,and specific transformation, with all carbon sources when the substrate concentration was increased from 0.08 mM to 0.2 mM probably due to substrate inhibition of the N-demethylase enzyme by codeine phosphate.

This occurred at a much lower concentration than the value of 1 mM found by Sewell in previous studies[18], with *C. echinulata*. More important, although the specific codeine transformations obtained with tetradecane cultures were higher than those obtained with glucose and succinate, the actual yield of norcodeine with tetradecane was similar to that obtained with the other carbon sources (see Figures 3.6 and 3.7). There was no advantage, therefore, in using a tetradecane culture with an increased codeine concentration to improve the transformation yield .

CHAPTER FOUR

THE N-DEMETHYLATION OF CODEINE BY CELL-FREE EXTRACTS PREPARED FROM *Cunninghamella* SPP.

Chapter 4. THE N-DEMETHYLATION OF CODEINE BY CELL-FREE EXTRACTS PREPARED FROM *CUNNINGHAMELLA* SPECIES

4.1 Introduction

In Chapter 3 various species of the fungus *Cunninghamella* demonstrated the ability to N-demethylate the test substrate, codeine, added to the medium in which the cells were growing. In order to characterise the enzyme(s) responsible for N-demethylation, it would be required to extract the enzyme(s) from the fungal cells to produce a cell-free extract which retained its N-demethylase activity. Various methods which have proved effective in liber - ating enzymes from microbial cells have been reported by several workers[106-107]. These methods generally involve mechanical rupture of the cell-wall and membrane by one or more of a combination of techniques, including hydraulic presses[108], sonication[109], mechanical shaking with abrasives[110] and mechanical or manual grinding[111]. The choice of procedure will depend on the ease with which the cell is ruptured, and also the sensitivity and the localisation of the enzyme within the cell. Mechanical rupture of the rigid cell wall may liberate readily soluble enzymes, but not enzymes which are membrane bound within the cell. Previous studies by Sewell[18] demonstrated that for the effective extraction of the N-demethylase enzyme from *C. echinulata* both a cell-wall disruption step, and also treatment with a detergent, Triton X100,was required in the extraction procedure. However, this method produced a crude cell-free extract, with a relatively low degree of N-demethylase activity.

The aim of this study was to improve on Sewell's original extraction procedure[18], and produce a cell-free extract from *Cunninghamella* species with a high degree of N-demethylase activity. Some properties of the extract could then be investigated including preliminary characterisation of the N-demethylase enzyme, and optimisation of the reaction conditions for codeine N-demethylation.

4.2 Materials

4.2.1 Chemicals and Reagents

Triton X100, Coomassie G250 dye, semicarbazide HCl (Analar), sodium dithionite (Analar), carbon-monoxide gas, 1,1,1-trichloroacetic acid (Analar), formaldehyde solution (Analar), sodium chloride (Analar), ferrous sulphate (Analar), calcium chloride, potassium chloride (Analar), ethylene diamine tetraacetic acid, sodium salt (EDTA) and polyethylene glycol (P.E.G.) were all purchased from B.D.H. Ltd.

Bovine serum albumin, urea, xanthine oxidase, NADPH (tetrasodium salt) and NADH (disodium salt) were purchased from Sigma London Chemical Co. Nitrogen gas (white spot); British Oxygen Ltd. Nash Reagent; this was prepared from ammonium acetate (112.5 g) acetylacetone (1.50 ml), acetic acid (2.25 ml) and distilled water

to 250 ml. All these reagents were of Analar grade except acetylacetone (Puriss grade, Koch-Light Laboratories Ltd.) which was distilled immediately before use. The Nash reagent was stored at 4°C and discarded if not used within one week. Phosphate buffer was prepared freshly before use from Analar grade reagents, KH_2PO_4 and Na_2HPO_4, BDH Ltd. The pH of each solution was checked with a pH meter. Coomassie G250 dye was prepared as a 0.06% solution in 3% perchloric acid (Fisons, UK) and was filtered through Whatman No. 1 filter paper to remove any undissolved material. The stock solution was diluted with 3% perchloric acid solution to give an absorbance of 1.3 - 1.5 at 465 nm, the absorbance maximum for the leuko form of the dye. The stock solution was stored at room temperature.

4.2.2 Equipment and instrumentation

Incubation tubes; Glass, 15 ml capacity with screw caps, Fisons Scientific Apparatus Ltd.

Centrifuge tubes; Polypropylene, 15 ml capacity with closures, MSE Scientific Instruments Ltd.

Water baths; Recirculating, Grant Instruments Ltd., Cambridge

Centrifuge; MSE High Speed 18, with 16 x 15 ml rotor, MSE Scientific Instruments Ltd.

Homogeniser; 4 blade with baffled glass chamber, 100 ml capacity; MSE Scientific Instruments Ltd.

Ballmill; Braun cell homogeniser, Model MSK, operated at a radial frequency of 2000 rpm with capillary coolant (CO_2) - Glasperlen.

Shaking bottle; with ground stopper and retainer, Pyrex, 75 ml, Glasperlen.

Glass beads; 0.45 - 0.5 mm diameter, Glasperlen.

All glassware used for the cell-free extract experiments was washed and brushed in tap water until visibly clean, then rinsed in glass distilled water and treated with chromic acid solution for 24 hours. The glassware was then rinsed five times with glass distilled water and finally soaked in fresh distilled water for 12 hours before drying at 45°C.

Before use, centrifuge tubes were washed in RBS-25 solution (Chemical Concentrates Ltd., London), and thoroughly rinsed with tap water and then distilled water. The tubes and caps were dried at 45°C.

4.3 Methods

4.3.1 Enzyme extraction procedures

Culture flasks were prepared and inoculated with *C. bainieri* or with *C. echinulata* according to the procedures and incubation conditions described in section 3.3.1 (page 133). The freshly harvested cells were washed with ice-cold 0.066 M phosphate buffer, pH 7.0, and then subjected to each of the enzyme extraction methods A - F described below. All extraction methods were conducted in a cold room at 3 - 4°C unless otherwise stated.

A. Homogenisation and agitation with Triton X100; Wet mycelia (20 g) was suspended in ice-cold 0.066 M phosphate buffer, pH 7.0 (28.5 ml) contained in a glass homogenisation vessel. The exterior of the vessel was packed with ice and the suspension was sheared at maximum speed for 3 minutes. An aqueous solution containing Triton X100 (10% v/v) in phosphate buffer (0.066 M, pH 7.0) was prepared and cooled to 4°C. The homogenate was transferred to a pre-cooled 125 ml Erlenmeyer flask to which the Triton X100 solution (1.5 ml) was added, giving a surfactant concentration of 0.5% w/v. The mixture was agitated on a radial shaker at 3 - 4°C for 30 minutes.

B. Pestle and mortar and milling; Wet mycelium (10 g) was ground to a paste with ice-cold 0.066 M phosphate buffer, pH 7.0 (10 ml) in a pestle and mortar. The paste was transferred to a pre-cooled pyrex shaking bottle (75 ml) containing 0.45 - 0.5mm diameter glass beads (50 g). The glass bottle was sealed, and shaken at 2000 rpm for two 30 second periods, inside a Braun Cell Homogeniser. The temperature of the glass bottle was maintained at 3 - 4°C by liquid carbon-dioxide cooling. Cell debris and the glass beads were removed by filtration through a stainless steel mesh (pore size 0.4 mm) and this was washed with phosphate buffer (2 x 10 ml).

C. Pestle and mortar, milling, and agitation for 30 minutes; Wet mycelium (10 g) was ground to a paste with ice-cold 0.066 M phosphate buffer, pH 7.0 (10 ml) in a pestle and mortar, and milled in the

Braun Cell Homogeniser as in (B) above. The cell debris and glass beads were removed by filtration (stainless steel mesh) and washed with phosphate buffer (2 x 10 ml). The combined filtrate and washings were transferred to a pre-cooled Erlenmeyer flask (125 ml) and agitated on a radial shaker at 3 - 4°C for 30 minutes.

D. Pestle and mortar, milling and agitation with Triton X100 (0.5% v/v) for 30 minutes; This method was as for method (C) above except for the addition of an aqueous solution containing Triton X100 (10% v/v) to the filtrate and washings in the pre-cooled Erlenmeyer flask (125 ml), to give a final concentration of 0.5% v/v, which was then agitated on a radial shaker at 3 - 4°C for 30 minutes.

E. Pestle and mortar, and agitation with Triton X100 (0.5% v/v) for 30 minutes; Wet mycelium (10 g) was ground to a paste with ice-cold 0.066 M phosphate buffer, pH 7.0 (10 ml) in a pestle and mortar. The paste was washed into a pre-cooled Erlenmeyer flask (125 ml) with ice-cold phosphate buffer (20 ml) and Triton X100 solution (10% v/v) was added to give a surfactant concentration of 0.5% v/v. The mixture was agitated on a radial shaker at 3 - 4°C for 30 minutes.

At the end of the extraction methods (A - E) described above the resultant suspension in each case was immediately centrifuged in polypropylene tubes (15 ml) at 27,000 g, 4°C for 20 minutes to remove the cell debris. The supernatant was decanted to

give a "cell-free extract".

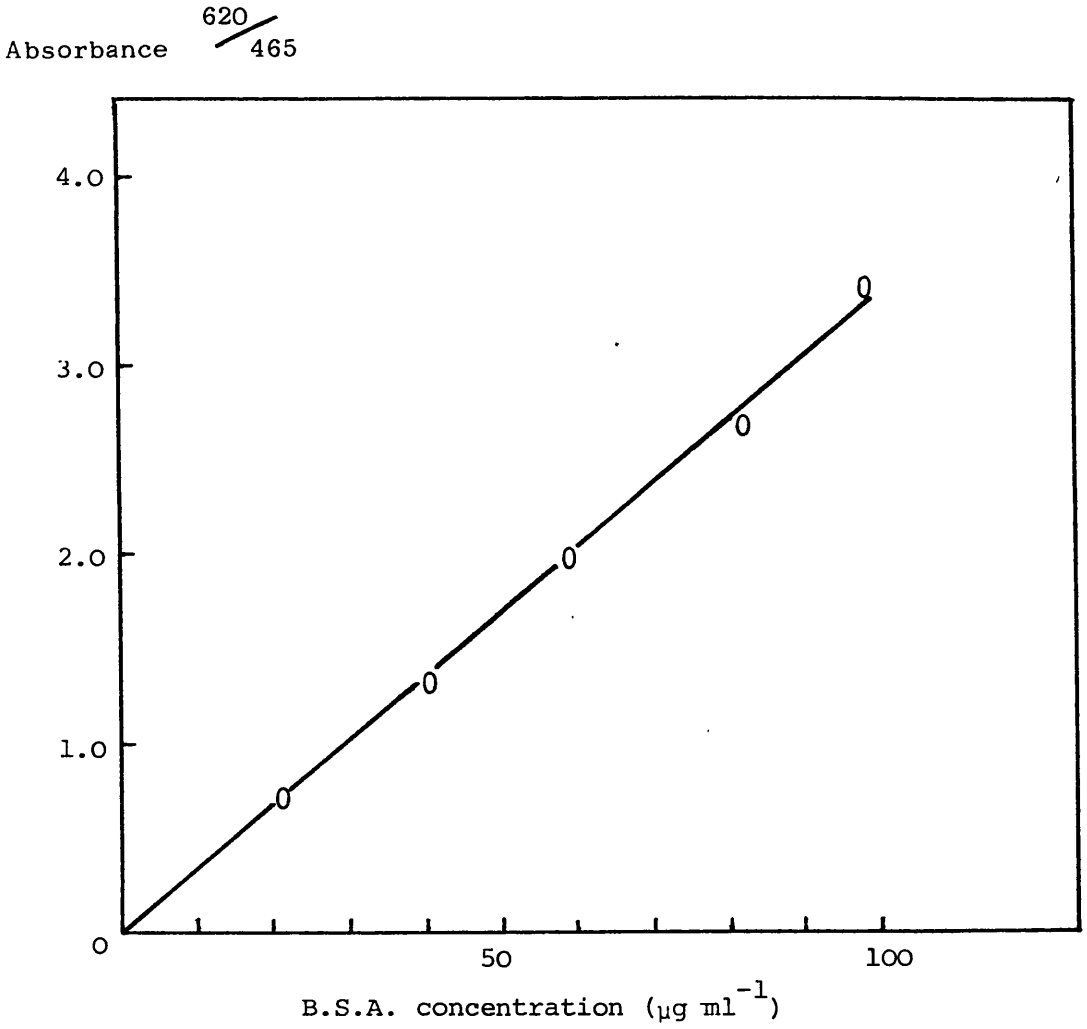
F. Dialysis of the cell-free extract; A piece of Visking tubing (20 cm) was boiled in an aqueous solution of ethylene diamine tetracetic acid (EDTA) (1%) for two minutes to remove calcium ions (Ca^{2+}). The tubing was tied at one end, cooled to 3 - 4°C, and cell-free extract was pipetted into it. This was then sealed with string, and immersed in a solution of ice-cold polyethylene glycol (P.E.G.), 10% w/v, in 0.066 M phosphate buffer, pH 7.0 contained in a conical flask (250 ml). The extract was left to dialyse in a cold room at 3 - 4°C for approximately 4 hours.

4.3.2 Determination of protein

Protein was determined by the method of Sedmak and Grossberg[112] employing Coomassie Brilliant Blue G250 dye, and Bovine Serum Albumin (B.S.A.) as the standard protein. This technique was reputed to suffer no interference from free amino acids and chemicals, such as Triton X100, that interfere with the Lowry protein assay[113]. However, to confirm this, the calibration curve was conducted in the presence of possible sources of interference such as codeine phosphate and Triton X100.

A series of standard solutions containing B.S.A. over a concentration range of 10 to 100 µg/ml in unbuffered saline (sodium chloride solution, 0.14 M), containing codeine phosphate (1 mM), Triton X100 (0.5% v/v) and urea (3 M) were prepared. The protein concentration was estimated by the method of Sedmak and Grossberg[112] in which an aliquot (0.5 ml) of each standard

Fig. 4.1. Calibration plot for protein in saline containing codeine phosphate (1 mM), Triton X100 (0.5%) and Urea (3 M)



solution was added to G250 solution (see page 156) in a stoppered tube and mixed immediately. Sodium chloride solution (0.5 ml) with B.S.A. omitted, mixed with G250 dye (0.5 ml) served as the control solution. The absorbance of each mixture was measured spectrophotometrically at 620 nm and 465 nm, against sodium chloride solution. Figure 4.1 shows the ratio of absorbance at 620/465 nm, as read against sodium chloride solution, plotted against the respective B.S.A. concentration, after the absorbance ratio of the 1:1 dye-sodium chloride solution mixture (control) had been subtracted from the values. This plot was then subjected to linear regression analysis:

slope	=	0.033
Intercept	=	-0.006
Correlation coefficient	=	0.997

The protein concentration in cell-free extracts was calculated from equation 4.1:

$$\text{protein concentration } (\mu g\ ml^{-1}) = \frac{\text{Absorbance } (^{620}/_{465}) - \text{control}}{0.033} \quad \ldots 4.1$$

4.3.3 Estimation of N-demethylase activity

The oxidative N-demethylation of codeine produced an equimolar quantity of formaldehyde and the secondary amine, norcodeine. The method of Nash[114] was used to determine the formaldehyde generated, since formaldehyde could arise from either the O-demethylation or

N-demethylation of codeine. A HPLC assay was employed to distinguish between O- and N- demethylation, and measure the N-demethylated product, norcodeine, directly.

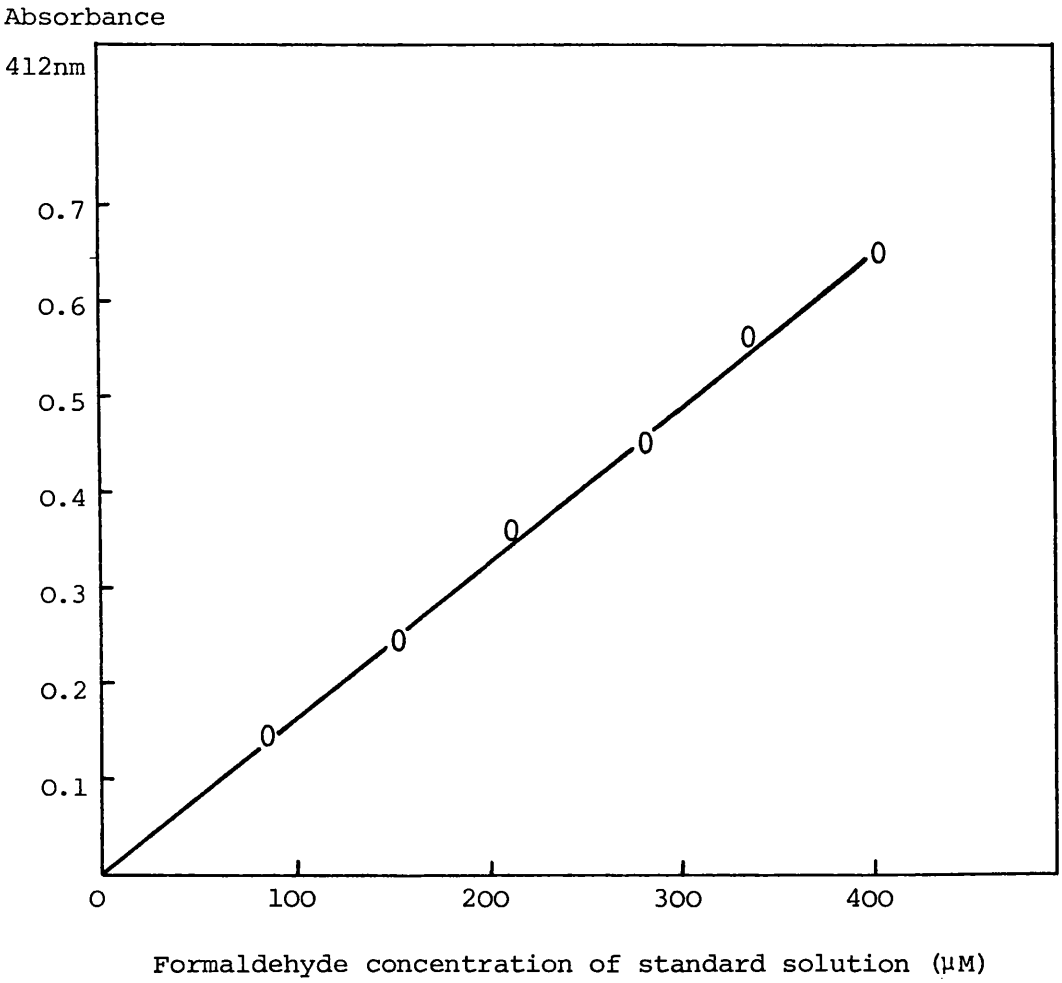
a) Determination of formaldehyde

One ml aliquots of the sample were pipetted into 15 ml polypropylene centrifuge tubes containing ice-cold 10% w/v 1,1,1-trichloroacetic acid (1.5 ml). The samples were centrifuged at 27,000 g, 4°C for 20 minutes, and volumes of the clear supernatant (2 ml) were transferred to glass screw cap tubes containing Nash reagent (1.0 ml). The samples were mixed and heated in a water bath at 60°C for 10 minutes. After cooling to room temperature, the absorbance of both test and control (omitting enzyme) samples was determined spectrophotometrically at 412 nm against a distilled water blank.

A standard curve was prepared for the assay of formaldehyde standard solutions incorporating any reaction mixture components which may interfere with the Nash reaction. A stock solution of formaldehyde (approximately 40% w/v HCHO) was standardised by the method of the British Pharmacopoeia[75]. This was used to prepare standard solutions of formaldehyde with concentrations ranging from 32.5 to 389.6 μM in 0.066 M phosphate buffer, pH 7.0, and also containing codeine phosphate (1.5 mM), semicarbazide HCl (3 mM), B.S.A. (1 mg ml^{-1}), Triton X100 (0.5% v/v), tetradecane (1% v/v) and urea (3 M).

The standard plot obtained is shown in Figure 4.2, and gave the

Fig. 4.2. Standard plot for the determination of formaldehyde by the Nash method [114]



following regression analysis results:

Slope	= 0.00160
Intercept	= -0.002
Correlation coefficient	= 0.999

The molar extinction coefficient for the Nash reaction product, 3,5-diacetyl-1,4-dihydrolutidine was 8051 $M^{-1}cm^{-1}$, which was within the suggested limits of ± 2.0%[149] for the calculated extinction coefficient of 8000 $M^{-1}cm^{-1}$. This indicates that the incubation mixture components did not interfere significantly with the Nash assay.

The formaldehyde in the test samples is given by Equation 4.2:

$$\text{Formaldehyde concentration } (\mu m) = \frac{(A_{test} - A_{control})}{0.00160} \quad \ldots 4.2$$

where A = absorbance at 412 nm.

b) Determination of norcodeine

The transformation sample (1.5 ml) was heated in a water bath at 100°C for 10 minutes, and then the mixture was pipetted into polypropylene centrifuge tubes (2.0 ml) capped, and centrifuged at 10,000 r.p.m. for 5 minutes. The supernatant was filtered through a 0.45 µm cellulose acetate membrane filter (Millipore), and 100 µl samples were assayed for norcodeine using the reversed phase HPLC method with ODS-Hypersil, 5 µm, described previously in Section 2.4.5

and the external standard procedure described on page 57.

The linearity of the detector response and the precision of response was reported in Section 2.4.5 for codeine and norcodeine samples in both mobile phase and transformation media. These values did not change significantly when the parameters above were redetermined using standard solutions of codeine and norcodine in transformation media, also containing Triton X100 (0.5% v/v), BSA (1 mg ml^{-1}) urea (3 M) and semicarbazide HCl (3 mM). This confirmed that the incubation mixture components do not interfere with the HPLC assay for norcodeine.

4.4 Experimental

4.4.1 Development and optimisation of a procedure for the extraction of the N-demethylase from *C. echinulata* and *C. bainieri*

a) Evaluation of the extraction methods A - E.

In order to assess the suitability of the various enzyme extraction methods described in section 4.3.2, (A - E),it was necessary to determine their efficiency under experimental conditions. The effectiveness of the extraction procedures was measured in terms of the protein concentration in the resulting cell-free extract, and also the N-demethylase activity of the extract on a codeine substrate.

Ten day cultures of *C. echinulata* and *C. bainieri* were harvested and subjected to the extraction method (A-E) described in Section 4.3.2. For each extraction method the following parameters were determined:- a) the weight of wet cells initially used for extraction, the protein concentration in the cell-free extract (by the procedure described in Section 4.3.3) and the N-demethylase activity of the resulting extract. N-demethylase activity was determined using the following incubation mixture; cell-free extract (1.3 ml), $FeSO_4.7H_2O$ (0.1 mM), NADPH (0.1 mM), NADH (0.1 mM), semicarbazide HCl (3 mM), and 0.066 M phosphate buffer, pH 7.0, to 1.5 ml. The mixtures were incubated at 30°C and the reaction initiated by the addition of codeine phosphate (1.5 mM) as substrate. Control mixtures in which codeine phosphate was omitted were also included. Samples of 1.0 ml were taken over the initial 30 minutes of incubation, and assayed for formaldehyde by the method described on page 163 . For the estimation of norcodeine the whole incubation mixture (1.5 ml) was subjected to the treatment described in Section 4.3.3 (b), page 165. The N-demethylase activity of the cell-free extract was expressed by 1) the specific activity defined in equation 4.3:-

$$\text{Specific activity } (\mu M\ min^{-1} mg^{-1}) = \frac{\text{Initial rate}}{\text{Protein in 1.3 ml extract}} \quad \ldots 4.3$$

where the initial rate (μ M min^{-1}) was obtained from the plot of formaldehyde produced in the incubation mixtures (μ M) against time, and also by 2) the total activity defined in Equation 4.4:-

$$\text{Total activity } (\mu\text{moles } \ell^{-1}) = \frac{\text{Max. norcodeine conc.}(\mu M) \times \text{total volume of extract (ml)}}{1.3(\text{ml})} \quad \ldots 4.4$$

where the maximum norcodeine concentration (in the incubation mixture) was that determined by HPLC.

The results obtained for the extraction of *C. echinulata* and *C. bainieri* by methods A - E are shown in Tables 4.1 and 4.2 respectively.

The highest yield of protein, together with the highest N-demethylase activity of the extracts produced from both *C. echinulata* and *C. bainieri* was obtained by method D (Table 4.1 and 4.2). This method consisted of grinding the cells in a pestle and mortar, milling the crude extract in a Braun Cell Homogeniser and then agitating the cell suspension with Triton X100 (0.5% v/v)) for 30 minutes.

b) The effect of Triton concentration, and agitation time, on the extraction of the N-demethylase from *C. echinulata* and *C. bainieri*

Further attempts were made to improve the enzyme extraction procedure by examining the effect of various surfactant concentrations and agitation times employed in Method D above. The effectiveness of each modification to the procedure was evaluated as in the previous section in terms of the protein yield in the resulting cell-free extract, and the N-demethylase activity on

Table 4.1. The extraction of the N-demethylase from *C. echinulata* by methods A - E

Extraction method	Protein per g wet cells	Total activity per g wet cells	Specific activity $\mu M\ min^{-1}\ mg^{-1}$
A. Homogenisation Agitation 30 mins with Triton X100 (0.5% v/v)	1.86	13.5	4.0
B. Pestle and mortar milling	1.7	3.8	1.9
C. Pestle and mortar milling Agitation (30 mins)	3.7	12.2	3.6
D. Pestle and mortar milling Agitation with Triton (30 min) (0.5% v/v)	15.7	46.3	19.3
E. Pestle and mortar Agitation with Triton (0.5% v/v, 30 mins)	1.84	4.3	2.3

Table 4.2. The extraction of the N-demethylase from *C. bainieri* by methods A- E

Extraction method	Protein per g wet cells	Total activity per g of wet cells	Specific activity $\mu M\ min^{-1} mg^{-1}$
A	2.8	32.3	14
B	1.92	5.8	1.6
C	5.6	27.6	16
D	27.2	107.7	34
E	1.84	4.3	0.95

a codeine substrate.

Ten day cultures of *C. echinulata* and *C. bainieri* were harvested and subjected to the pestle and mortar and milling procedures described for Method D above. The combined filtrate and washings obtained from separate extractions were combined with Triton X100 solution (10% v/v) in pre-cooled Erlenmeyer flasks (125 ml) to give a range of surfactant concentrations in the final mixture from 0 to 3% v/v. A mixture containing no Triton X100 was included as a control. The mixtures were agitated on a radial shaker at 3 - 4°C for 30 minutes, and then centrifuged at 27000 g, 4°C for 20 minutes in polypropylene centrifuge tubes to remove the cell-debris. The protein yield in resulting cell-free extracts was estimated by the procedure described in Section 4.3.3, and the N-demethylase activity on a codeine substrate by the HPLC method described in Section 4.3.4(b). Plots of protein yield per wet cell weight, total activity per wet cell weight and specific activity all against Triton X100 concentration (% v/v) were constructed, and are illustrated in Figure 4.3.

The results in Figure 4.3 showed that the amount of protein extracted increased with the Triton X100 concentration used in the extraction procedure. However, the data in Figure 4.3 also showed that concentrations of Triton X100 greater than about 0.5% caused a significant reduction in the N-demethylase activity of the cell-free extracts produced. The implications of these observations are discussed on page 195. However, it was concluded from these results that the optimum Triton X100 concentration was 0.33% v/v.

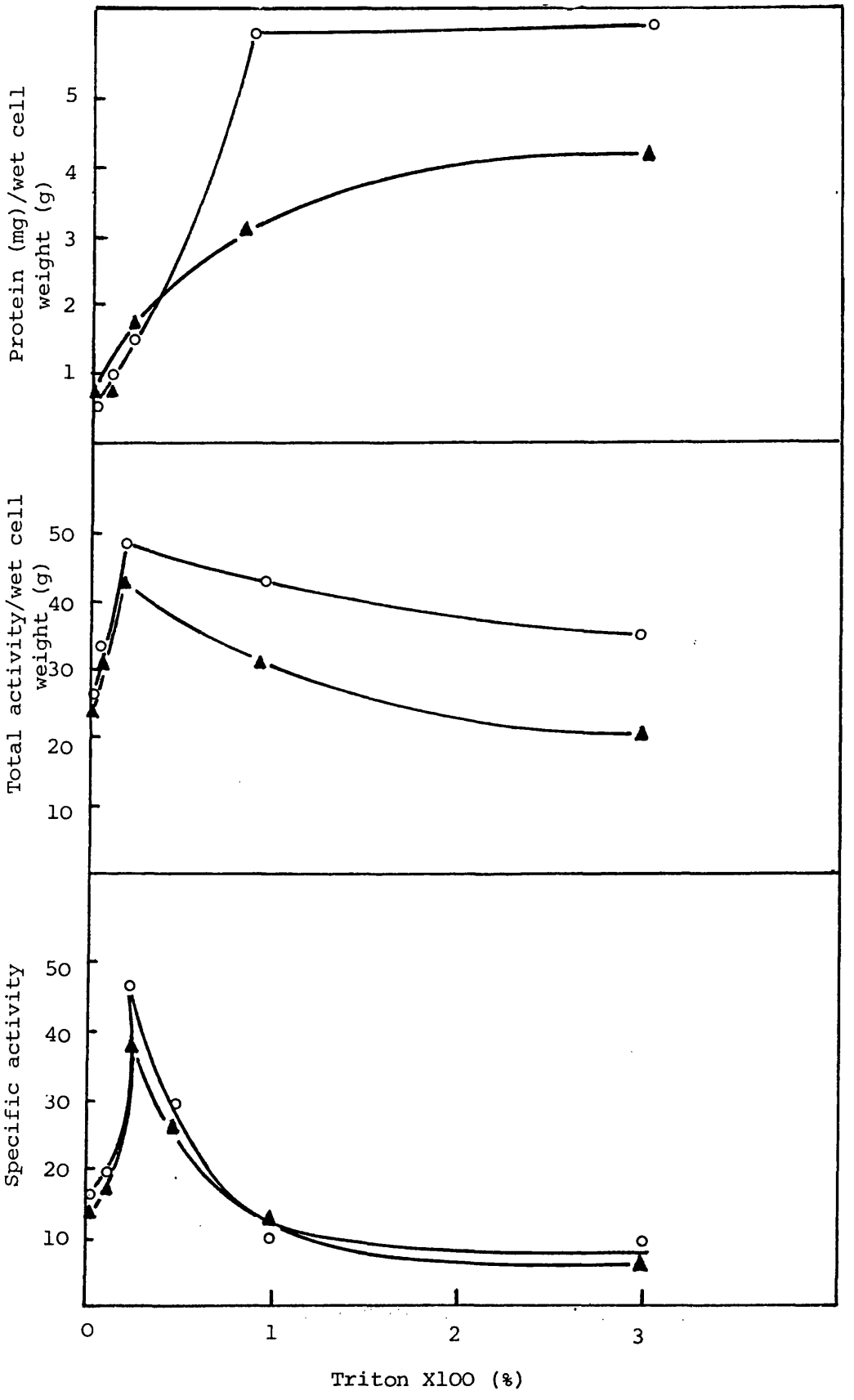


Figure 4.3. The protein yield, total activity and specific activity of cell-free extracts from *C . bainieri*o and *C. echinulata* ▲ with various concentrations of Triton X100 used in the extraction procedure

The extraction procedure was therefore repeated using a Triton X100 concentration of 0.33% v/v and varying the agitation time from 0 minutes (no agitation) to 60 minutes. Since both micro-organisms, *C. echinulata* and *C. bainieri* gave similar results the results for the extraction of *C. bainieri* only are shown in Table 4.3 below.

Table 4.3. The effect of agitation time with Triton X100 (0.33%) on the extraction of the N-demethylase from *C. bainieri*.

Agitation time (minutes)	Protein (mg) per g wet cells	Specific Activity $\mu M\ min.^{-1}\ mg^{-1}$
0	0.92	12
15	2.2	27
30	2.7	34
45	2.5	22
60	2.6	18

The amount of protein extracted, and also the N-demethylase activity of the extracts, reached an optimum at approximately 30 minutes agitation with Triton X100 (0.33% v/v). After this time there was no significant increase in the protein extracted, and the N-demethylase activity actually decreased. Therefore, agitation for 30 minutes with a Triton X100 concentration of 0.33% v/v was considered to be the optimum combination, and was incorporated into method D (page 159) and used in all subsequent enzyme extraction experiments. Furthermore, it was observed

from the extraction studies that cell-free extracts prepared from *C. bainieri* generally exhibited higher N-demethylase activities than those prepared from *C. echinulata*. In view of this, *C. bainieri* only was used in all further studies.

c) The effect of urea on the extraction of the N-demethylase from *C. bainieri*

The N-demethylase in *Cunninghamella* sp. could be membrane associated or membrane bound. Membrane associated proteins are not usually bound directly to the hydrocarbon portion of the lipid bilayer, and are readily dislodged from their membranes and solubilised by chelating agents or by lowering or increasing the ionic strength of the pH, without dissociating the lipid matrix of the membrane[115]. Alternatively, the enzyme could be more tightly bound to the membrane. If this was the case it may only be solubilized by disrupting the membrane with detergents eg. Triton X100[116]. The nature of the N-demethylase in *C. bainieri* was investigated by extracting cultures with urea, and comparing the extraction results with those obtained with Triton X100.

Ten day cultures of *C. bainieri* were harvested and subjected to extraction method D. This procedure was repeated so that in the final stage of the method the crude extract was agitated with either (a) Triton X100 at a concentration of 0.33% v/v, or with (b) urea at a concentration of 3 M. The procedure was also repeated with no additive to the crude extract, as a control. Protein in

the cell-free extract was estimated as before (page 160) and the N-demethylase activity of the cell-free extract determined by the HPLC assay for norcodeine (page 79). The results obtained are shown in Table 4.4.

Table 4.4. The effect of urea (3 M) and Triton X100 (0.33%) on the extraction of N-demethylase from *C. bainieri* by extraction method D.

Treatment	Protein in cell-free extract mg/g wet cells	Specific Activity $\mu M.min^{-1}mg^{-1}$
Agitation 30 mins (control)	0.35	15
Agitation 30 mins + urea (3 M)	0.50	17
Agitation 30 mins + Triton X100 (0.33% v/v)	2.3	38

From the results in Table 4.4 the highest protein yield together with the highest N-demethylase activity was obtained using Triton X100 in the extraction procedure. The results with urea were only slightly higher than the control results, indicating that urea was ineffective at solubilizing the enzyme which was probably membrane bound.

4.4.2 Enrichment of the N-demethylase enzyme from cell-free extracts of *C.bainieri* by dialysis

The cell-free extract produced by method D (page 159) consists of a mixture of the N-demethylase enzyme, plus other components extracted from the fungal cells, in phosphate buffer. The dialysis procedure (F) described in Section 4.3.1 (f) was performed in an attempt to remove aqueous medium from the extract to enrich the N-demethylase enzyme in the resulting extract.

Ten day cultures of *C. bainieri* were harvested and subjected to the modified extraction method D procedure described on page 172, using 0.33% v/v Triton X100 and an agitation time of 30 minutes. A known volume of the resulting cell-free extract was then subjected to the dialysis procedure (F) (page 160) using dialyais tubing with an exclusion limit of 6000 molecular weight. The extract was allowed to dialyse at 3 - 4°C for about 4 hours. After this time the extract was recovered and the final volume measured. The protein concentration, was determined in both the dialysed and undialysed extracts. In addition the N-demethylase activity of dialysed and undialysed extracts was estimated on a codeine substrate as in Section 4.4.1, and expressed as the specific transformation;

$$\text{specific transformation} = \frac{\text{Norcodeine concentration } (\mu M)}{\text{Protein (mg) in 1.3 ml extract}} \quad \ldots 4.5$$

The results obtained are shown in Table 4.5 .

Table 4.5. The protein concentration and N-demethylase activity of cell-free extracts from *C. bainieri* before and after dialysis.

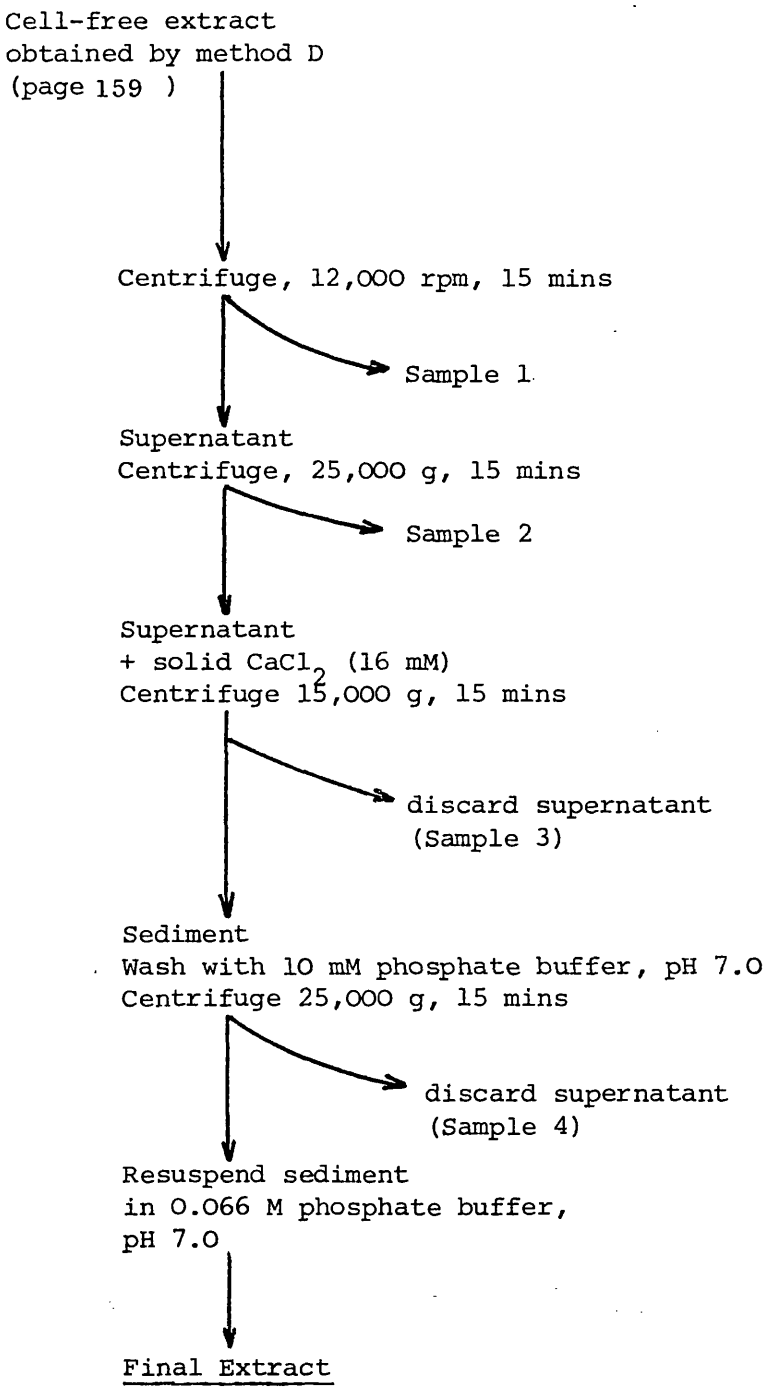
Treatment	Protein concentration (mg ml^{-1}) in extract	Specific transformation
Undialysed	0.225	20.2
Dialysed	0.95	6.1

The results in Table 4.5 show that the protein concentration in the cell-free extract was increased by approximately a factor of 5 by dialysis, but the specific transformation decreased. This suggests that essential components necessary for the microbial N-demethylation process to occur may have been removed from the extract by dialysis. Therefore there was no advantage in employing this procedure in future extraction experiments.

4.4.3 Enrichment of the N-demethylase enzyme from cell-free extracts of *C. bainieri* by calcium chloride precipitation

An attempt was made to isolate an active fraction from the cell-free extract of *C. bainieri* produced by method D (page 159.) by adapting the method of Kapelli *et al.*[117] This involved the precipitation of the microsomal fraction from *C. bainieri* cell-free extract by the addition of calcium chloride ($CaCl_2$). A diagram showing the stages of the procedure is shown in Figure 4.4.

Figure 4.4. Scheme for the enrichment of the N-demethylase enzyme from cell-free extracts of *C. bainieri* by calcium chloride precipitation



The cell-free extract obtained from *C. bainieri* by extraction method D, page 159 ,was immediately centrifuged in polypropylene tubes (15 ml) at 12000 g, 4°C for 15 minutes. The supernatant was then centrifuged at 25,000 g, 4°C for 15 minutes. Solid calcium chloride ($CaCl_2$) was added to the supernatant to a concentration of 16 mM, and the microsomal fraction collected by a further centrifugation at 15,000 g, 4°C for 15 minutes. The supernatant at this stage was discarded, and the sediment was washed once with KCl (150 mM) in 10 mM phosphate buffer, pH 7.0 (a volume equal to the volume of supernatant discarded), and then the mixture centrifuged once more at 25,000 g, 4°C for 15 minutes. The final pellet was resuspended in 0.066 M phosphate buffer, pH 7.0, to give the purified cell-free extract.

Samples (1 to 5) were taken from each stage of the purification procedure as shown in Figure 4.4 and analysed for the protein yield, and the N-demethylase activity against a codeine substrate.

The results obtained are shown in Table 4.6.

Table 4.6. Protein yield (mg) and specific transformation values determined for samples 1 - 5 obtained from the enrichment of cell-free extracts of *C. bainieri* by calcium chloride precipitation (Figure 4.6).

Sample	Protein yield (mg)	Specific Transformation
1	12.9	18.2
2	8.4	14.8
3	6.1	11.5
4 (washing stage)	-	-
5	0.7	30.1

The results in Table 4.6 show that there was a progressive decrease in protein yield in samples 1 to 5 taken at each stage of the calcium chloride enrichment procedure. However the specific transformation values in extracts 1 to 5 significantly increased indicating that the enrichment technique was successful. However, the overall procedure was considered to be too time consuming for the routine production of cell-free extracts for experiments described in this chapter.

4.4.4 Confirmation of N-demethylase activity from the incubation of cell-free extracts of *C. bainieri* and *C. echinulata* with codeine

The method of Nash[114] was used to estimate the formaldehyde generated from the incubation of codeine with cell-free extracts of *Cunninghamella* sp. It was assumed that the formaldehyde was generated from the microbial transformation of codeine. However, it could have arisen from the chemical degradation of codeine, or have been released from the cell-free extract (endogenous formaldehyde) under the test conditions.

The following control incubation mixtures were prepared to determine the origin of the formaldehyde generated in the test assay.

Mixture A) $FeSO_4.7H_2O$ (0.1 mM), NADPH (0.1 mM), NADH (0.1 mM), semicarbazide HCl (3 mM) and 0.066 M phosphate buffer, pH 7.0, to 1.5 ml.

Mixture B) $FeSO_4.7H_2O$ (0.1 mM), NADPH (0.1 mM), NADH (0.1 mM),

semicarbazide HCl (3 mM) and 0.066 M phosphate buffer, pH 7.0, to 0.2 ml.

To confirm whether or not the formaldehyde was generated from the chemical degradation of codeine, duplicate incubation mixtures (A) were incubated at 30°C, and codeine phosphate substrate was added to give a concentration of 1.5 mM in the mixture. Samples of 1 ml were taken immediately (0 minutes) and after 30 minutes incubation, and assayed for formaldehyde by the Nash method (page 163). There was no significant difference in the quantity of formaldehyde detected at zero and 30 minutes incubation indicating that formaldehyde was not produced from the chemical breakdown of codeine in the test assay.

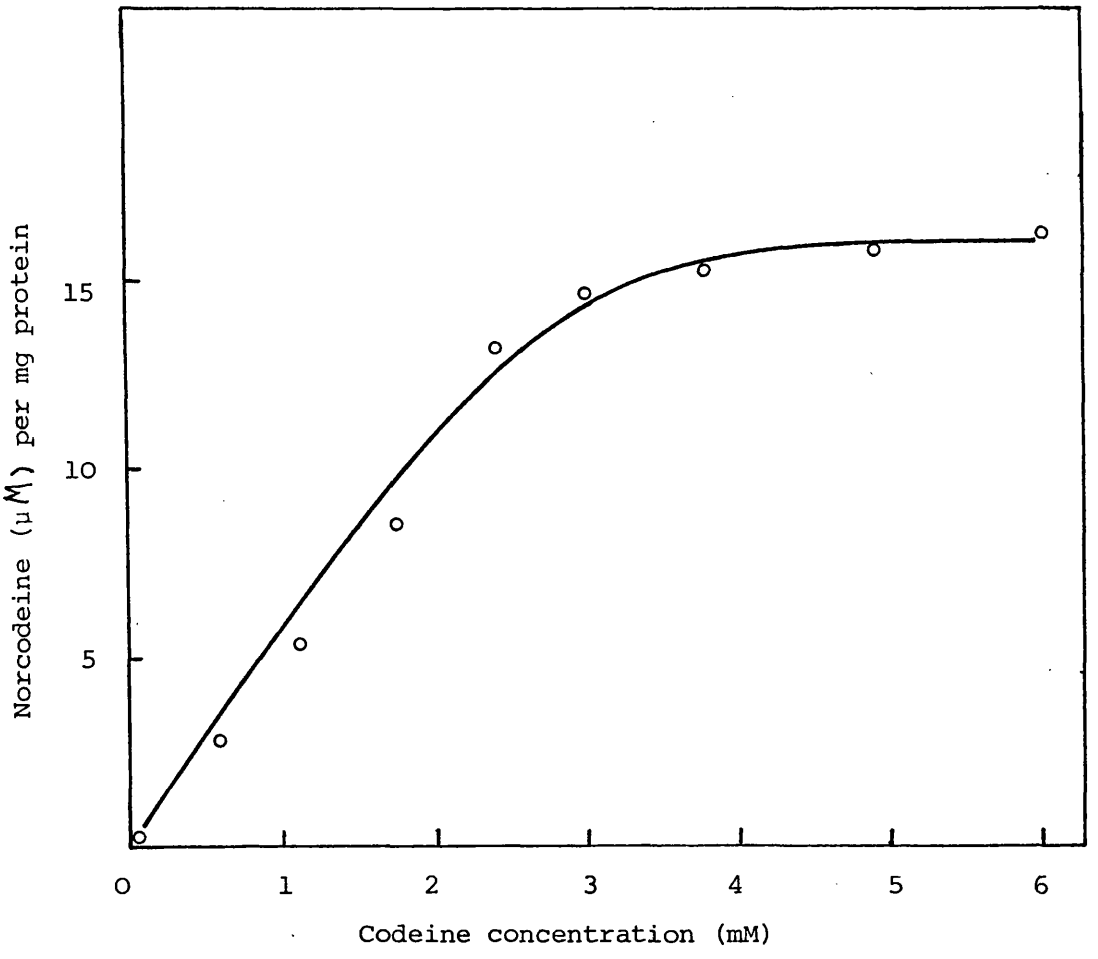
Cell-free extract (1.3 ml) was added to a series of incubation mixtures (B), and incubated at 30°C. Samples of 1.0 ml were taken over the initial 30 minutes of incubation, and assayed for formaldehyde as before. There was no significant increase in the quantity of formaldehyde found in the incubation mixtures over the 30 minute incubation period. This indicated that endogenous formaldehyde was not released from cell-free extracts during the course of the incubation procedure. The results of both sets of control experiments ensured that the formaldehyde was generated from the microbial transformation of codeine. However, this could have arisen from either the O-demethylation of codeine to produce morphine or the N-demethylation of codeine to produce norcodeine. Therefore, incubation mixtures containing cell-free extract, test mixture components and codeine were incubated as before and samples

taken for HPLC analysis, employing the ODS-Hypersil method (Section 2.4.5). The chromatograms obtained conclusively demonstrated the presence of norcodeine, and the absence of morphine in the incubation mixture. This indicates that the formaldehyde generated by the cell-free extract arose only from the N-demethylation of codeine.

4.4.5 The effect of substrate concentration on the transformation of codeine by cell-free extracts from *C. bainieri*

In all the cell-free extract experiments performed so far in this chapter the codeine concentration in the incubation mixtures was 1.5 mM. This codeine concentration was employed by Sewell[18] to study the transformation of codeine by cell-free extracts from *C. echinulata*, although no attempt was made to find if it was optimal. Therefore, the effect of codeine concentration on the transformation of codeine, by cell-free extracts from *C. bainieri* was investigated. The cell-free extract was prepared from ten day cultures of *C. bainieri* employing extraction method D described in Section 4.3.1. Incubation mixtures were prepared of the following composition: cell-free extract (1.3 ml); $FeSO_4.7H_2O$ (0.1 mM), NADPH (0.1 mM), NADH (0.1 mM), semicarbazide HCl (3 mM) in 0.066 M phosphate buffer, pH 7.0, (0.2 ml). The mixtures (1.5 ml) were incubated at 30°C, and the reaction initiated by adding codeine phosphate to provide a concentration range from 0 mM to 6.0 mM in the final mixtures. After 30 minutes the reaction was terminated by boiling the mixtures for 10 mimutes at 100°C. The protein concentration of the incubation mixtures was determined, in addition to the HPLC determination of norcodeine, and the transformation

Figure 4.5. The effect of codeine concentration on codeine transformation by cell-free extracts of *C. bainieri* at 30°C, pH 7.0.



activity was expressed as the norcodeine produced (μM) per mg of total protein. These values were plotted against the codeine concentration in the incubation mixtures as shown in Figure 4.5.

The amount of codeine N-demethylated increased with codeine concentration up to a concentration of approximately 3 mM in the incubation mixture. At higher concentrations there was no significant increase in codeine N-demethylation, and so a codeine concentration of 3 mM was employed in all subsequent test assay incubation mixtures described in this thesis.

4.4.6 The effect of added co-factors on codeine transformation by cell-free extracts from *C. bainieri*

The co-factors NADPH and NADH and ferrous iron (Fe^{2+}) have all been implicated as essential requirements for several microbial monooxygenase catalysed reactions[22,23,25]. In each case the concentration reported to be optimal in previous studies[25] was 0.1 mM. The effect on codeine transformation, by cell-free extracts from *C. bainieri* of NADPH, NADH and Fe^{2+} addition at concentrations of 1×10^{-4} M was initially investigated.

a) The effect of Fe^{2+}

Incubation mixtures were prepared each containing the components shown in Table 4.7.

Table 4.7. Composition of incubation mixtures used to study the effect of added Fe^{2+} on codeine transformation by cell-free extracts from *C. bainieri*

Cell-free extract (ml)	Components added to phosphate buffer in each mixture	0.066 M phosphate buffer, pH 7.0 (ml)
0.5	Codeine phosphate (3mM)	1.0
0.75	Semicarbazide HCl (3mM)	0.75
1.0	$FeSO_4$ $7H_2O$ (0.1mM)	0.5
1.3		0.2
0.5	Codeine phosphate (3mM)	1.0
0.75	Semicarbazide HCl (3mM)	0.75
1.0	(controls)	0.5
1.3		0.2

b) The effect of NADPH and NADH

Incubation mixtures were prepared each containing codeine phosphate (3 mM) and semicarbazide HCl (3 mM) and the components shown in Table 4.8. Each incubation mixture was allowed to equilibriate to 30°C, and the reaction was initiated by the addition of codeine phosphate. After 30 minutes the reaction was terminated by boiling each mixture for 10 minutes. Samples of each mixture were assayed for protein and norcodeine, and the transformation activity expressed as the norcodeine produced (μM) per mg of total protein. The values obtained for both sets of incubation mixtures were plotted against the volume of cell-free extract used and are shown in Figures 4.6 and 4.7 respectively.

Table 4.8. Composition of incubation mixtures used to study the effect of added co-factors on codeine N-demethylation by cell-free extracts from *C. bainieri*

Cell-free extract (ml)	Components added to phosphate buffer in each mixture	0.066 M phosphate buffer pH 7.0 (ml)
0.5		1.0
0.75	Codeine phosphate (3mM) Semicarbazide HCl (3mM)	0.75
1.0	(controls)	0.50
1.3		0.20
0.5		1.0
0.75	Codeine phosphate (3mM) Semicarbazide HCl (3mM)	0.75
1.0	NADPH (0.1mM)	0.50
1.3		0.20
0.5		1.0
0.75	Codeine phosphate (3mM) Semicarbazide HCl (3mM)	0.75
1.0	NADH (0.1mM)	0.50
1.3		0.20
0.5		1.0
0.75	Codeine phosphate (3mM) Semicarbazide HCl (3mM)	0.75
1.0	NADPH (0.1mM) NADH (0.1mM)	0.50
1.3		0.20

Figure 4.6. The effect of added Fe^{2+} on codeine transformation by cell-free extracts from *C. bainieri* at 30°C, pH 7.0

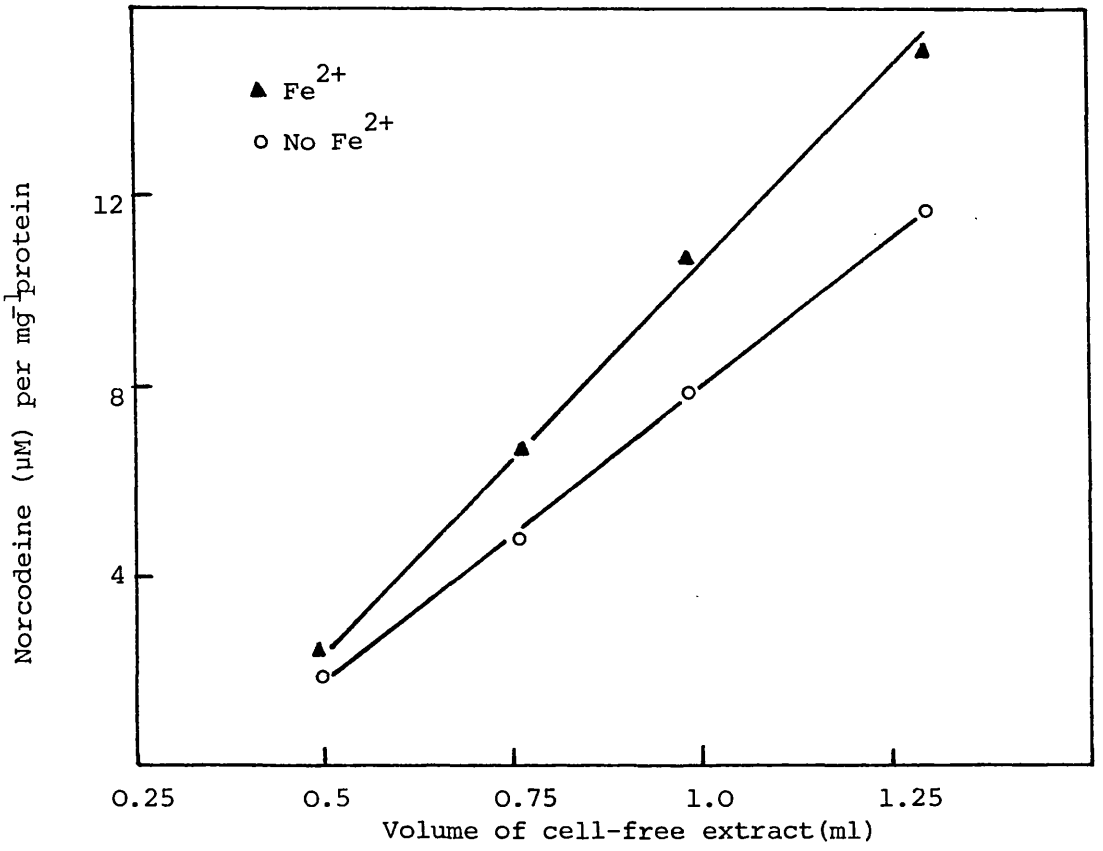
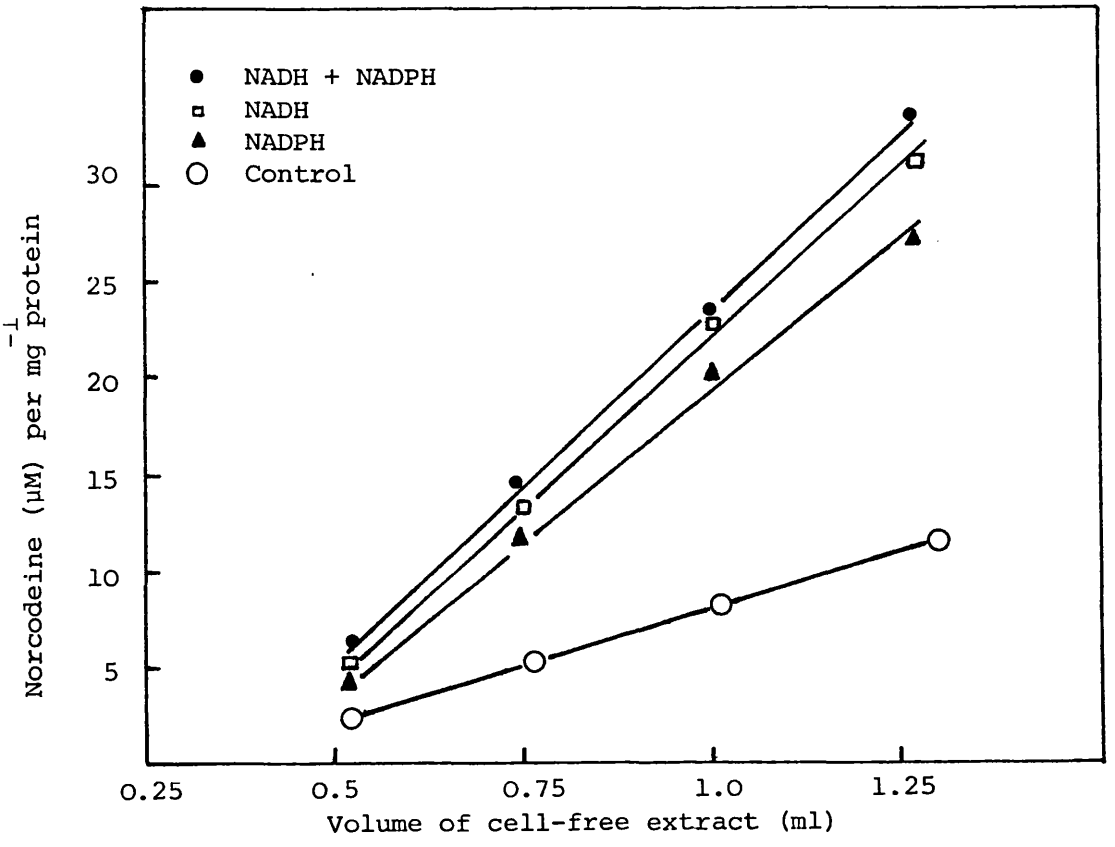


Figure 4.7. The effect of added NADPH and NADH on codeine transformation by cell-free extracts from *C. bainieri* at 30°C, pH 7.0.



The addition of Fe^{2+}, NADH and NADPH (each at a concentration of 0.1 mM) resulted in significant increases in the amount of codeine N-demethylated. The addition of NADH resulted in the highest N-demethylase activity, although there was a slight improvement found when both NADPH and NADH were added together. The above investigation was carried out using only one co-factor concentration (0.1 mM). Further experiments were therefore carried out to observe the effect of various co-factor (NADH) concentrations on the N-demethylation of codeine.

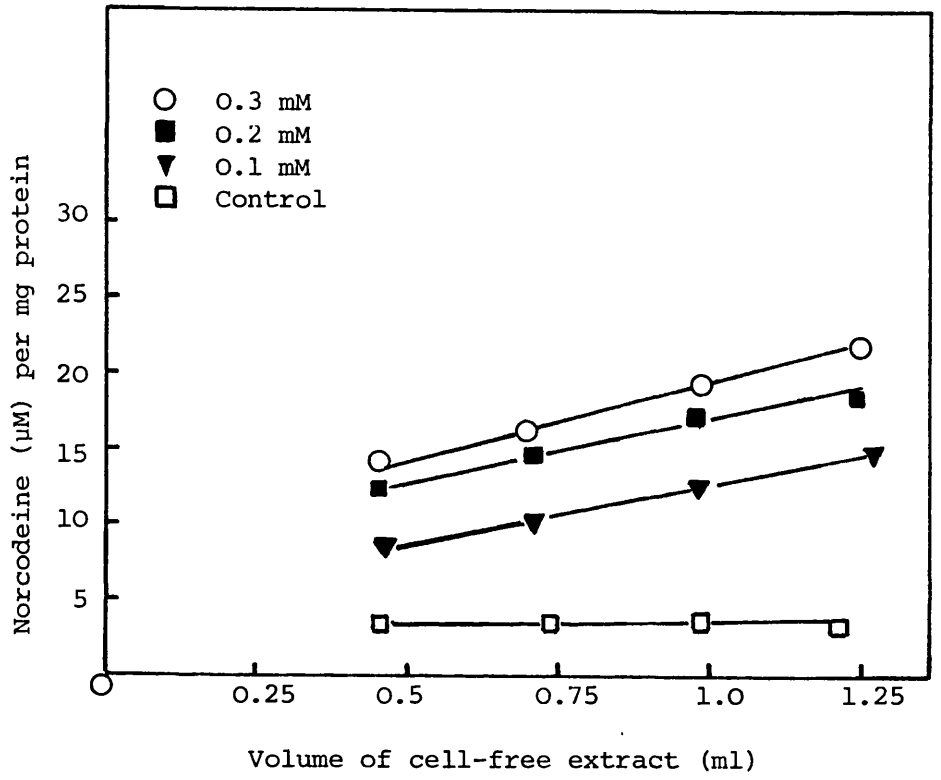
Incubation mixtures were prepared each containing codeine phosphate (3 mM) and semicarbazide HCl (3 mM) and also the components shown in Table 4.9.

Each incubation mixture was equilibriated at $30^{o}C$, and the reaction was initiated by the addition of codeine phosphate. Samples of each mixture were taken after 30 minutes incubation and assayed for norcodeine and protein, and the transformation activity determined as described above. Plots of norcodeine produced (μM) per mg protein against volume of extract used for each co-factor concentration and the control mixtures (no co-factor added) are shown in Figure 4.8.

Table 4.9. Composition of incubation mixtures used to study the effect of co-factor concentration on codeine N-demethylation by cell-free extracts from *C. bainieri*

Cell-free extract (ml)	Components added to phosphate buffer in each mixture	0.066 M phosphate buffer, pH 7.0 (ml)
0.5	NONE (controls)	1.0
0.75		0.75
1.0		0.5
1.3		0.2
0.5	NADH (0.1mM)	1.0
0.75		0.75
1.0		0.5
1.3		0.2
0.5	NADH (0.2mM)	1.0
0.75		0.75
1.0		0.5
1.3		0.2
0.5	NADH (0.3mM)	1.0
0.75		0.75
1.0		0.5
1.3		0.2

Figure 4.8. The effect of NADH concentration on codeine transformation by cell-free extracts from *C. bainieri* at 30°C, pH 7.0



The addition of increasing concentrations of NADH from 0.1 mM to 0.3 mM showed only a small increase in the amount of codeine N-demethylated, and so the NADH and NADPH concentrations used in all subsequent enzyme experiments remained at 0.1 mM.

4.4.7 Investigation of substrate and co-factor requirements on codeine transformation by cell-free extracts of *C. bainieri*

It was observed from previous experiments that the amount of codeine N-demethylation in incubation mixtures with cell-free extracts

from *C. bainieri* was limited to about 4 - 5% maximum. The following experiment was designed to find out if this was due to a co-factor requirement, or due to an equilibrium imbalance between codeine and norcodeine in the incubation mixture.

Cell-free extract (1.0 ml) prepared from 10 day cultures of *C. bainieri*, employing extraction method D, was incorporated into 5 similar incubation mixtures each containing the following:- codeine phosphate (3 mM), semicarbazide HCl (3 mM), $FeSO_4.7H_2O$ (0.1 mM), and 0.066 M phosphate buffer, pH 7.0 to 1.5 ml. A sixth tube was also included containing all the test mixture components except codeine (control). The mixtures were allowed to equilibriate at 30°C, and the reaction was initiated by the addition of codeine phosphate. Samples (0.5 ml) were taken from each mixture after 30 minutes incubation, and assayed for norcodeine by HPLC. Further additions were made to the remaining incubation mixtures as shown in Table 4.10.

These second incubation mixtures were incubated again for 30 minutes at 30°C, after which samples were taken from each mixture and assayed for norcodeine. The concentration of norcodeine in each of the mixtures after the first and second incubation periods are shown in Table 4.10.

Table 4.10. Components added to produce the second incubation mixtures, and the norcodeine concentration (μM) in the mixtures after the first and second incubations at 30°C for 30 minutes as described above.

Tube	Components added to produce the second incubation mixtures	Norcodeine in the incubation mixtures (μM) first incubation	second incubation
1	Cell-free extract (1 ml)	25.4	51.9
2	Codeine phosphate (3 mM) in phosphate buffer (1 ml)	23.6	24.9
3	Phosphate buffer (1 ml)	22.6	22.3
4	Cell-free extract pre-treated with xanthine oxidase (1 ml)	21.9	22.1
5	Cell-free extracted pre-treated with boiled xanthine oxidase (1 ml)	22.6	45.1
6	Phosphate buffer (1 ml)	None	None

After the first 30 minute incubation period the results in Table 4.10 show that codeine was N-demethylated to norcodeine to approximately the same extent in tubes 1 to 5. Norcodeine was not detected in the control mixture (tube 6) containing no codeine. Incubation of a second portion of extract added to the original incubation mixture (tube 1) resulted in the doubling of the norcodeine concentrations. However, the addition of more codeine (tube 2), or more buffer, to dilute the original codeine present in the mixture (tube 3), both failed to increase the norcodeine

concentration. These observations suggested that an equilibrium between codeine and norcodeine in the mixtures was not the reason for the limited transformation.

The concentration of norcodeine remained constant after the addition of a cell-free extract pretreated with xanthine oxidase (tube 4), an enzyme capable of deactivating co-factors such as NADPH and NADH. Whereas, the concentration of norcodeine was doubled when the xanthine oxidase was first boiled before adding it to the cell-free extract (tube 5). These results suggest that the N-demethylation reaction was limited by the availability of endogenous co-factors such as NADH or NADPH in the cell-free extract incubation mixture.

4.5 Discussion

A suitable procedure was required to extract the N-demethylase from the fungal cells of *Cunninghamella* in order to characterise the enzyme. The extraction methods investigated (A - E Section 4.3.1) were evaluated in terms of the protein concentration in the resulting cell-free extract, and the N-demethylase activity of the extract on a codeine substrate. Several of the extraction procedures described incorporated the non-ionic surfactant, Triton X100, a substance known to interfere with the Lowry protein assay[113]. It was possible to remove the Triton by adsorption onto Amberlite beads[118], but this was a time-consuming procedure. In practice, it was more convenient to use the Coomassie

Blue protein assay[112] (Section 4.3.3) in which Triton X100 did not interfere. The Coomassie Blue assay for proteins in solution depends on the conversion of Coomassie Brilliant Blue G250 dye in dilute acid from a brownish-orange (the leuko form) to an intense blue colour when the dye-anion interacts with the NH_3^+ groups of proteins. Absorbance of the dye-protein mixture is measured spectrophotometrically. The assay is reputed to have high reproducibility and to be more sensitive than the Lowry protein assay[112].

The results obtained from extraction methods (A - E) performed on harvested cells of *C. echinulata* and *C. bainieri* are shown in Table 4.1 and 4.2 respectively. Extraction method A was that developed by Sewell[18] to prepare crude cell-free extracts from *C. echinulata*. Homogenisation was found to effectively disrupt the fungal pellets, and the non-ionic surfactant, Triton X100, was included for the solubilisation of protein associated with the cellular membrane fragments[18]. The additional methods B to E in Tables 4.1 and 4.2 were attempts to improve on Sewell's original extraction procedure,(Method A). Grinding the mycelium in a pestle and mortar, and milling the crude extract (method B), was found to effectively disrupt the fungal pellets but the protein yield and the N-demethylase activity of the final cell-free extract was relatively low. Agitation of the crude extract (produced by method B above) for 30 minutes in phosphate buffer, (method C), doubled the protein yield in the final extract, but there was no improvement in the N-demethylase activity over Sewell's original method (A).

However, agitation of the crude extract (produced by method B) for 30 minutes in the presence of Triton X100 (method D) considerably enhanced both the protein yield, and N-demethylase activity of the resulting extract, confirming the importance of including Triton X100 in the extraction procedure. Furthermore, the relatively low extraction results obtained with method E provided further evidence that initially an effective disruption process was required, prior to the Triton X100 treatment, for the best results. Grinding with the pestle and mortar failed to efficiently disrupt the fungal cells, and hence produced low extraction results, even after the crude extract was agitated with Triton X100. Employing extraction method D, the protein yield and resulting N-demethylase activity were 8 - 10, and 2 - 3 times greater than values obtained by Sewell's original extraction procedure (A) for the extraction of *C. echinulata* and *C. bainieri*

The effect of varying the surfactant (Triton X100) concentration and agitation time, on the extraction of *C. bainieri* and *C. echinulata* using extraction method D, produced the results shown in Figures 4.3, and Table 4.3 respectively. Increasing the Triton X100 concentration from 0 to 3% produced a significant increase in protein yield in the final extract. However, the N-demethylase activity of the extract actually decreased with increasing Triton concentrations above 0.5% v/v, implicating that the Triton X100 may be exerting a deleterious effect on the N-demethylase enzyme. A Triton X100 concentration of 0.33% v/v was found to be optimal, and employing this concentration, the optimum extraction results

were obtained by agitating the mixture for at least 30 minutes, after which time there was no further effect. Furthermore, the protein yield and N-demethylase activity of extracts prepared by method D incorporating Triton X100 (0.33%), were considerably higher than those obtained with urea (3 M). This suggested that the N-demethylase enzyme was tightly bound to the membrane (membrane bound) as opposed to being simply membrane associated. Triton X100 has been used previously to solubilize a number of membrane bound proteins, including membrane enzymes[119,120], without loss of their biological activities[121]. It has been proposed[122] that Triton X100 functions by solubilization of enzymes with an amphiphilic structure consisting of a water-soluble enzymic moiety and a hydrophobic moeity which is bound to the cell membrane. Triton X100 is capable of solubilizing the hydrophobic part of the enzyme preventing its adsorption to hydrophobic surfaces[122]. The effect of increasing detergent concentration on the solubilization of membrane bound proteins has been studied by Tanford and Reynolds[116]. They found that at low detergent to membrane ratios, the single chain amphiphile will partition between the aqueous solution and the lipid-protein bilayer. As the detergent to membrane ratio is increased, some protein-lipid complexes may be released from the membrane with bound detergent, and at very high detergent to membrane ratios lipid detergent mixed micelles and protein detergent complexes are formed, resulting in separation of the lipid from the protein[115]. Many membrane bound enzymes have been found to depend on specific lipids for activity[123,124], and this may be one of the reasons why Triton X100 concentrations above 0.5% used in the extraction process

profoundly affected the activity of the N-demethylase enzyme. Loss of N-demethylase activity may also have resulted from the denaturation of the N-demethylase enzyme at higher Triton X100 concentrations. Yoshida and Kumaoka[125] found, for example, that a monooxygenase isolated from *Candida tropicalis* showed a significant loss of biological activity at surfactant concentrations above 1% v/v. Denaturation was thought to be caused by a complex formed between the non-ionic surfactant and the monooxygenase enzyme[125].
An attempt to enrich the N-demethylase enzyme in cell-free extracts prepared by extraction method D by dialysis (Section 4.4.2) resulted in an increase in the protein yield, but with a significant reduction in N-demethylase activity. The activity could not be recovered by the addition of Fe^{2+}, NADPH and NADH to the dialysed extract indicating that other essential components for N-demethylation must have been dialysed out of the original extract.

Kapella *et al.*[117] demonstrated a procedure for the isolation of an enriched hydroxylase enzyme from the yeast *Candida tropicalis* by precipitation of the microsomal fraction with calcium chloride. Employing this procedure with cell-free extracts from *C. bainieri* (Section 4.4.3) successfully provided an enriched final extract, with a high degree of N-demethylase activity. However, the procedure was considered impracticable for routine experimental use because it was time-consuming and produced only small quantities of purified cell-free extract.

The use of control incubation mixtures in which codeine, and/or the cell-free extract were omitted (Section 4.4.4) provided evidence

that formaldehyde was generated from the microbial transformation of codeine. Furthermore, HPLC analysis revealed that only norcodeine was present in the reaction mixtures, implicating that the formaldehyde generated by the cell-free extracts resulted from the N-demethylation of codeine, as opposed to O-demethylation. Preliminary characterization of the cell-free extract prepared from *C. bainieri* by extraction method D was carried out from incubation experiments, with a codeine substrate. Incubation temperatures of 30°C, and a pH of 7.0, were used, as previous studies by Sewell[18] showed these to be optimal for the N-demethylation of codeine using cell-free extracts from *C. echinulata*. The transformation of codeine by *C. bainieri* was found to increase up to a concentration of 3 mM codeine in the incubation mixtures with 1.3 ml of cell-free extract; and this codeine concentration was used in all subsequent extract experiments. The addition of Fe^{2+} (see Figure 4.6), NADH and NADPH (see Figure 4.7) to the incubation mixture were all found to be necessary for significant codeine N-demethylation by *C. bainieri*. The inclusion of NADH resulted in a higher codeine N-demethylation than NADPH, but the highest codeine transformation was obtained by adding both NADH and NADPH together, implicating that a synergistic effect may be occurring. Increasing the NADH concentration in the incubation mixture from 0.1 mM to 0.3 mM resulted in corresponding increases in the amounts of codeine N-demethylated. However, these increases were not significant enough to warrant using higher concentrations in subsequent incubation experiments. The co-factors NADH and NADPH have been reported as essential requirements for both mammalian[22,23] and microbial

monooxygenase[24,25] catalysed reactions, serving as active electron donors. Ferrous iron has also been reported[26] to play an important role in the function of cytochrome P-450 linked monooxygenases and is believed to involve the Fe^{2+} mediated reduction of molecular oxygen in the cytochrome-substrate complex, thus permitting the incorporation of an oxygen atom into the substrate. The co-factor requirements described above for codeine N-demethylation by *C. bainieri*, suggest that the reaction is probably mediated by a type of monooxygenase enzyme. Control experiments performed in Section 4.4.7 showed that the limiting factor for codeine transformation in incubation mixtures with *C. bainieri* was not an unfavourable equilibrium between codeine and norcodeine, as increasing and decreasing the codeine concentrations in the mixture failed to improve transformation. The probable reason for low transformations was because endogenous co-factors present in the cell-free extract were utilised in the reaction and were not replenished. Since the identity of the endogenous co-factor was not known, it was not possible to add them to the incubation mixture to further improve the transformation yield.

CHAPTER FIVE

INVESTIGATION OF THE MECHANISM OF MICROBIAL N-DEMETHYLATION OF CODEINE BY CELL-FREE EXTRACTS OF *C. bainieri*.

CHAPTER 5. INVESTIGATION OF THE MECHANISM OF MICROBIAL N-DEMETHYLATION OF CODEINE BY CELL-FREE EXTRACTS OF *C. BAINIERI*

5.1 Introduction

Over the past 30 years or so the *in vivo* N-, O- and S- dealkylation of a wide variety of drugs and foreign compounds has been demonstrated in a variety of mammalian species[22]. In many cases dealkylations of this type have been attributed to cytochrome P-450 linked monooxygenases[23]. These enzyme systems exhibited a wide substrate specificity and required the presence of molecular oxygen and the reduced cofactor NADPH (reduced nictoninamide adenine dinucleotide phosphate). The feature of cytochrome P-450, which by definition was diagnostic for the presence, and presumed activity of this enzyme, was a characteristic absorption peak at 450 nm, observed as a U.V. difference spectrum in the presence of carbon monoxide in the test cuvette only. Sodium dithionite was added to both cuvettes of the recording spectrophotometer so that the haem iron present in the enzyme was fully in its reduced form (ferrous)[126].

The presence of monooxygenases of the P-450 type have also been reported in several micro-organisms which effect the oxidative metabolism of a variety of organic compounds[127]. A bacterial monooxygenase, for example, was described[128] in *Pseudomonas putida* which effected the oxidation of camphor, and contained an enzyme ($P\text{-}450_{cam}$) which was similar in many respects to the cytochrome P-450 present in mammalian systems. The structure of this

cytochrome P-450 was reported[129] to resemble a cytochrome P-450 isolated from rabbit liver microsomes with a similar subunit molecular weight and amino acid composition, but very different substrate specificity and solubility. In general, the substrate specificity of bacterial monooxygenases appears to be more limited than mammalian monooxygenases[25]. However, fungi and yeasts have demonstrated oxidative capabilities comparable to those effected by mammalian systems. Smith and Rosazza[130] having studied the comparative metabolism of xenobiotics by mammalian and microbial systems, first coined the term, "microbial models of mammalian metabolism" to describe the close parallel between the two sytems[130]. Several examples in which complex organic substrates have been transformed by fungi and yeasts have been reviewed by the same authors[131]. Ferris *et al.*[25,132] have demonstrated a fungal monooxygenase system in *Cunninghamella bainieri* (ATCC 9244) capable of carrying out reactions similar to those of mammalian liver systems. When used as a cell-suspension, supplemented with NADPH, this culture was shown to carry out the N-demethylation of aminopyrene, the O-demethylation of p-nitroanisole and anisole, the aryl hydroxylation of anisole, aniline and naphthalene and the reduction of both 1,2-dimethyl- 4 -(p-carboxyphenylazo)-5-hydroxy-benzene and p-nitrobenzoic acid[132]. These reactions were inhibited by carbon monoxide, SKF-525A and metapyrone, but not cyanide, and the requirement of molecular oxygen was proved by $^{18}O_2$ incorporation experiments. Furthermore, Ferris *et al.* later provided evidence for the presence of cytochrome P-450[25]. The fungal enzyme extract from *C. bainieri* (ATCC 9244) was solubilized in aqueous digitonin

followed by dithionite reduction and the addition of CO to give a maximum U.V. absorbance at 450 nm, characteristic of cytochrome P-450.

The possible mechanism(s) for the oxidative N-dealkylation of substrates containing tertiary amine functions, by both mammalian and microbial enzyme systems, has been a subject of debate over recent years, largely because the reaction intermediates have been transient and have not been observed. Essentially two routes of tertiary amine oxidation have been proposed for this process[133]. These are illustrated in Figure 5.1. In the first, primary oxidative attack is thought to occur upon the carbon α to the nitrogen (α-C oxidation) to give a carbinolamine, N-dealkylation may proceed directly *via* carbinolamine formation by an oxene insertion reaction, or alternatively, the substrate may undergo dehydrogenation to an iminium ion which then adds the equivalent of hydroxide to form the carbinolamine. The carbinolamine then cleaves due to its instability, and forms the secondary amine and the corresponding carbonyl product (Figure 5.1). The second mechanism proposes metabolic attack on the nitrogen (N-oxidation) to produce an N-oxide which yields the corresponding secondary amine and carbonyl products. Additionally, tertiary amine N-oxides are readily reduced to the parent drug[134,135] and some may undergo a rearrangement to the corresponding α-carbinolamine[136].

The ability of various species of the fungus *Cunninghamella*

cytochrome P-450 was reported[129] to resemble a cytochrome P-450 isolated from rabbit liver microsomes with a similar subunit molecular weight and amino acid composition, but very different substrate specificity and solubility. In general, the substrate specificity of bacterial monooxygenases appears to be more limited than mammalian monooxygenases[25]. However, fungi and yeasts have demonstrated oxidative capabilities comparable to those effected by mammalian systems. Smith and Rosazza[130] having studied the comparative metabolism of xenobiotics by mammalian and microbial systems, first coined the term, "microbial models of mammalian metabolism" to describe the close parallel between the two sytems[130]. Several examples in which complex organic substrates have been transformed by fungi and yeasts have been reviewed by the same authors[131]. Ferris *et al.*[25,132] have demonstrated a fungal monooxygenase system in *Cunninghamella bainieri* (ATCC 9244) capable of carrying out reactions similar to those of mammalian liver systems. When used as a cell-suspension, supplemented with NADPH, this culture was shown to carry out the N-demethylation of aminopyrene, the O-demethylation of p-nitroanisole and anisole, the aryl hydroxylation of anisole, aniline and naphthalene and the reduction of both 1,2-dimethyl- 4 -(p-carboxyphenylazo)-5-hydroxy-benzene and p-nitrobenzoic acid[132]. These reactions were inhibited by carbon monoxide, SKF-525A and metapyrone, but not cyanide, and the requirement of molecular oxygen was proved by $^{18}O_2$ incorporation experiments. Furthermore, Ferris *et al.* later provided evidence for the presence of cytochrome P-450[25]. The fungal enzyme extract from *C. bainieri* (ATCC 9244) was solubilized in aqueous digitonin

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The ability of various species of the fungus *Cunninghamella*

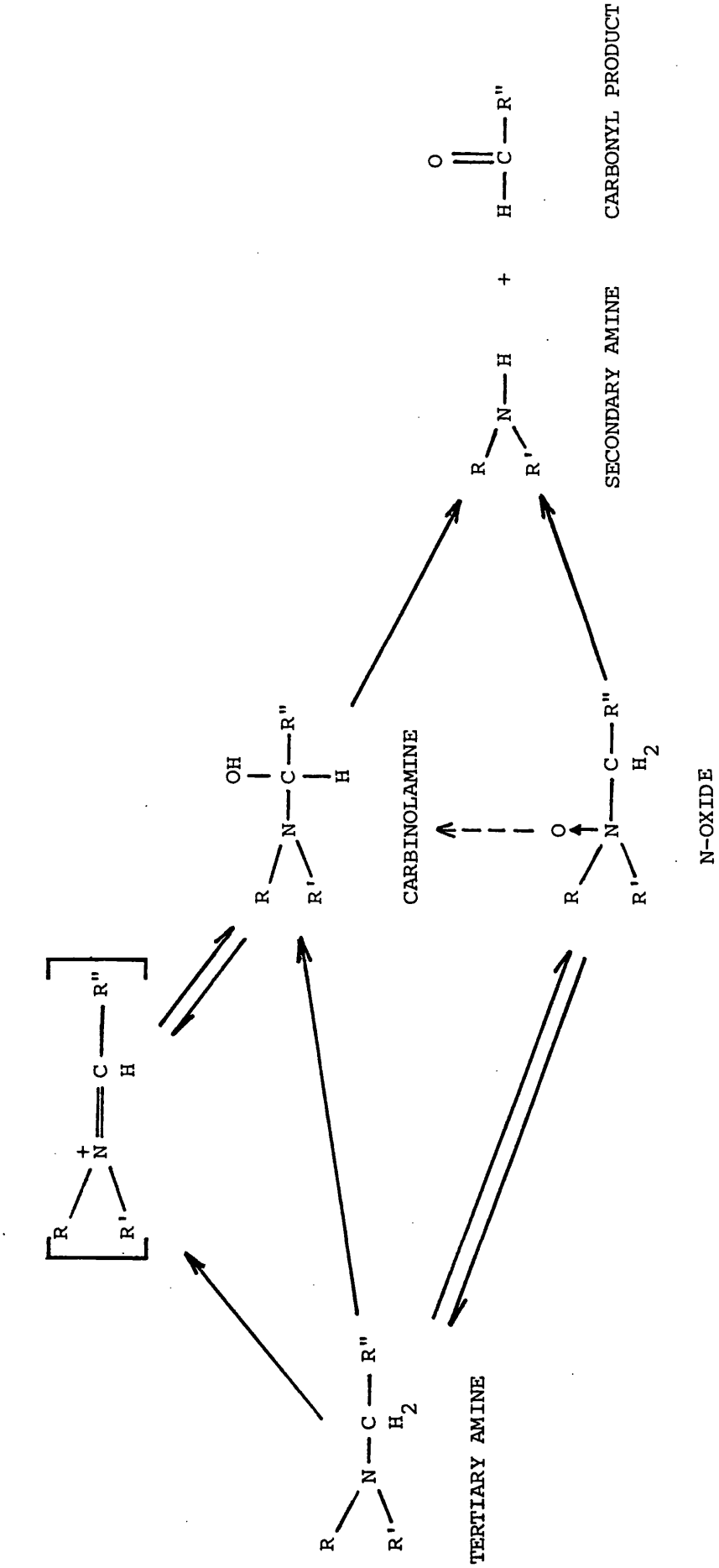


Figure 5.1. Possible mechanisms for the N-dealkylation of tertiary amines

to N-demethylate the tertiary amine, codeine, was demonstrated by Sewell[18], and also previously in Chapter 3 of this thesis.

It was demonstrated in Chapter 4 of this thesis that for codeine N-demethylation to occur by cell-free extracts of *C. bainieri*, the co-factors NADPH, NADH and iron (Fe^{2+}) were required to be present in the reaction mixture. The aim of this study, therefore, was to prepare cell-free extracts of *C. bainieri* to N-demethylate codeine and codeine N-oxide substrates, and estimate the N-demethylase activity in each case using the quantitative HPLC assay (developed in Chapter 2) to determine the transformation product, norcodeine. The technique would be used to determine kinetic data for the N-demethylation of codeine and codeine N-oxide substrates, and to investigate the affect of selected inducers and specific enzyme inhibitors for the same transformations. The evidence gained from these experiments could then be used to propose a reaction mechanism for the N-demethylation of codeine by cell-free extracts of *C. bainieri*.

5.2 Materials

5.2.1 Chemicals and Reagents

Hydrogen peroxide (100 volumes) - Fisons Ltd.

Palladium (Pd) on carbon (10% w/w) - Aldrich Chemical Co.

SKF 525A (proadifen hydrochloride); Smith Kline and French Ltd.

Potassium cyanide, sodium sulphite (AnalaR), 2-mercaptoethanol and L-Cystine- BDH Ltd.

5.2.2 Test compounds

Codeine N-oxide; was prepared from codeine base by the method of Groutas *et al.*[137] 30% Aqueous hydrogen peroxide (10 ml) was added to codeine base (4.8 g; 16 mmol.) in methanol (20 ml) contained in a 100 ml round bottomed flask. The solution was stirred for 48 hours at room temperature, and then heated on a steam bath for 2 h. Excess peroxide was destroyed by adding 10% Pd on carbon (0.1 g) and stirring the cooled reaction mixture for 1 hour. The reaction mixture was filtered (Whatman No. 1 filter paper) and the solvent removed by evaporation under reduced pressure to leave a white solid (3.6 g). This was dissolved in the minimum of hot ethanol, filtered and allowed to cool to room temperature to yield white crystals of codeine N-oxide (1.8 g).
Analytical; melting point 213°C (lit.[78] 214°C).
^{1}H NMR H^{δ} (D_2O, codeine N-oxide); 1.21 (2H, t, C_{15}-H); 1.85 (1H, d, C_{14}-H); 3.09 (2H, d, C_{10}-H); 3.39 (3H, m. C_{16}-H, C_9-H) C_9-H); 3.76 (1H, s, O-H, exchanges with D_2O); 3.84 (3H, s, O-Me); 4.34 (1H, m, C_6-H); 4.74 (3H, s, N-Me); 5.07 (1H, d, C_5-H), 5.35 (1H, m, C_7-H); 5.72 (1H, d, C_8-H); 6.79 (d.d, C_1-H, C_2-H).

Diazepam: Roche Products Ltd.

5.2.3 Equipment and Instrumentation

Neutral glass ampoules; 2 ml capacity, Epsom Glass Industries Ltd., England. All glassware used for the cell-free extract experiments was prepared according to the procedures described in Section 4.2.2 (page 156).

5.3 Methods

5.3.1 General methods

Culture flasks were prepared and inoculated with *C. bainieri* according to the procedures and incubation conditions described in Section 3.3.1. Cell-free extracts were prepared from 10 day cultures of *C. bainieri* employing extraction method D described in Section 4.3.2. Extracts were stored at $4^{o}C$ in an atmosphere of nitrogen until required for use. Protein in the cell-free extract was determined by the method of Sedmak and Grossberg[112] described in Section 4.3.2. N-demethylase activity of the cell-free extract was determined by assaying the norcodeine produced in incubation mixtures described in Section 4.4.1, page 166 by the HPLC procedure described in Section 4.3.3(b).

5.3.2 Estimation of cytochrome P-450

Each cell-free extract was subjected to the standard method of Omura and Sato[126] to detect the presence of cytochrome P-450.

Two matched quartz cuvettes were filled with cell-free extract, and nitrogen gas was bubbled through the extract in each cuvette

for 2 minutes. Solid sodium dithionite, $Na_2S_2O_4$, (10 mg) was added to each cuvette, and a linear baseline spectrum was measured from 380 nm to 520 nm on the UV/visible spectrophotometer with a full scale deflection of 0.1 absorbance units. Carbon monoxide was then bubbled through the sample cuvette for 30 seconds, and the spectrum retraced from 380 nm to 520 nm. An absorption maximum in the 450 nm region suggested the presence of cytochrome P-450, and the cytochrome P-450 concentration was calculated from the absorbance difference $A_{450} - A_{490}$ on the assumption that the *Cunninghamella* cytrochrome P-450 has the same absorption coefficient as reported by Omura and Sato[126] for liver microsomal cytochrome P-450 of 91 cm^{-1} mM^{-1}.

5.4 Experimental

5.4.1 Determination of the stability of the N-demethylase enzyme in the cell-free extract of *C. bainieri*

It was required to determine the stability of the cell-free extract to ensure that there was no appreciable loss of N-demethylase activity during the time required to conduct kinetic experiments.

Cell-free extract, prepared from codeine induced cultures of *C. bainieri* was divided into 1.5 ml aliquots and sealed in 2 ml glass ampoules. Prior to sealing, half of the ampoules were purged with a stream of nitrogen gas for 2 minutes to displace the air, and the remainder were sealed in air. The ampoules sealed under air and nitrogen were divided once more, and some of each type stored in a fridge at 4°C and the others in a freezer at -20°C.

At intervals during a 10 day period, ampoules were taken from storage, opened and the N-demethylase activity of the cell-free extract determined by the procedure described in Section 4.4.1 page 166 using codeine phosphate as the test substrate. The N-demethylase activity of unstored cell-free extract was assumed to be 100%, and the activities of stored extracts were determined with respect to this value. From these results plots of the N-demethylase activity of extracts stored in various conditions with storage time were constructed and shown in Figure 5.2. From these results it was observed that the activity of all the extracts decreased to approximately 70% of the original activity after 60 - 70 hours' storage. However, the overall trend was that extracts stored in atmospheres of nitrogen, maintained a higher % activity over the period of storage than extracts stored in air, but there was no significant difference between storage at $4^{o}C$ and storage at $-20^{o}C$.

5.4.2 The effect of reducing agents on the stability of cell-free extracts from *C. bainieri*

An attempt was made to improve the stability of the cell-free extract of *C.bainieri* during storage by the addition of selected reducing agents. Extracts were sealed in 2 ml glass ampoules as described above in the presence of one of the following reducing agents: sodium sulphite (1 mM), L-cystine (5 mM) or 2-mercapto-ethanol (5 mM). The extracts were stored at $4^{o}C$ in a nitrogen atmosphere. Samples were removed at regular time intervals over

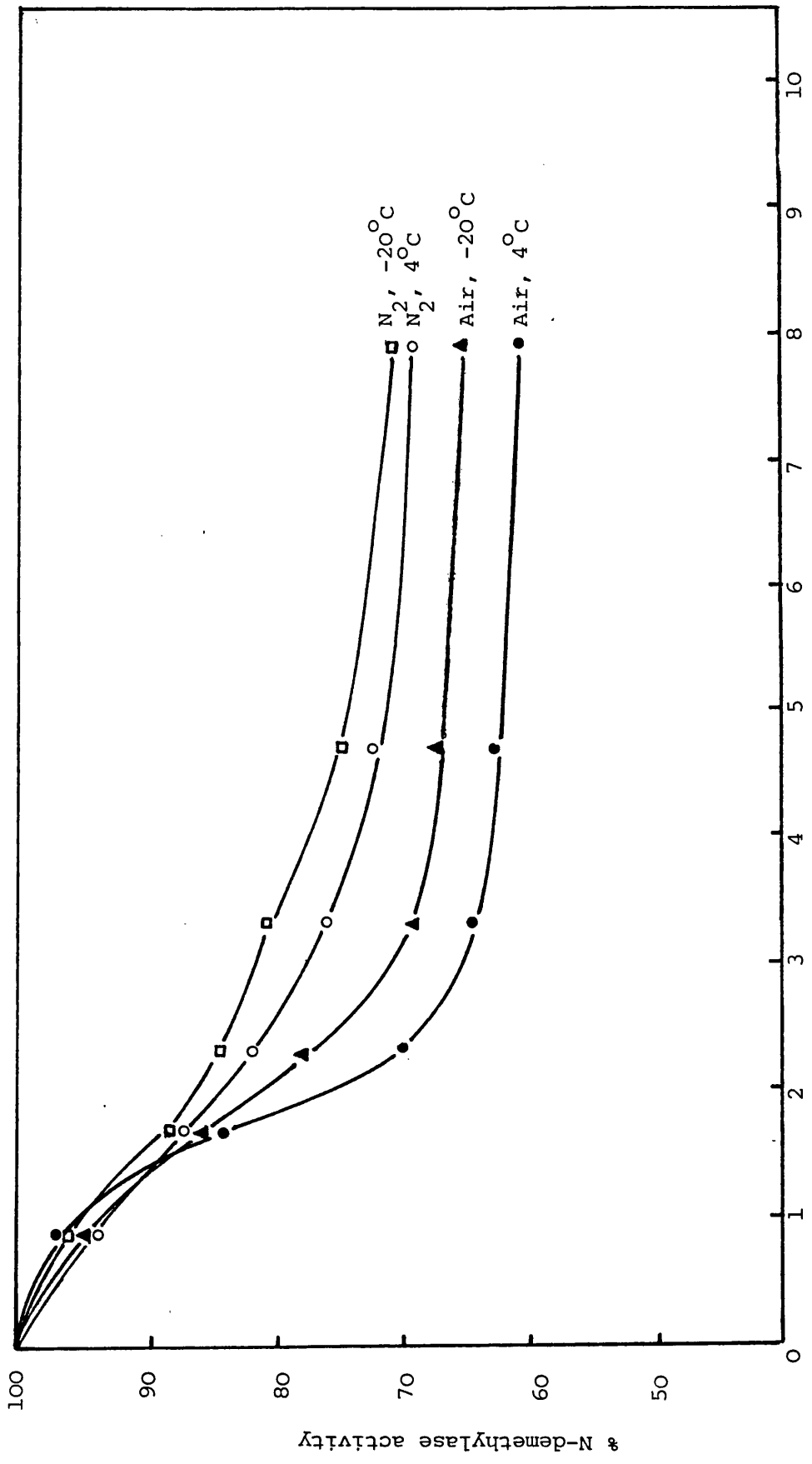
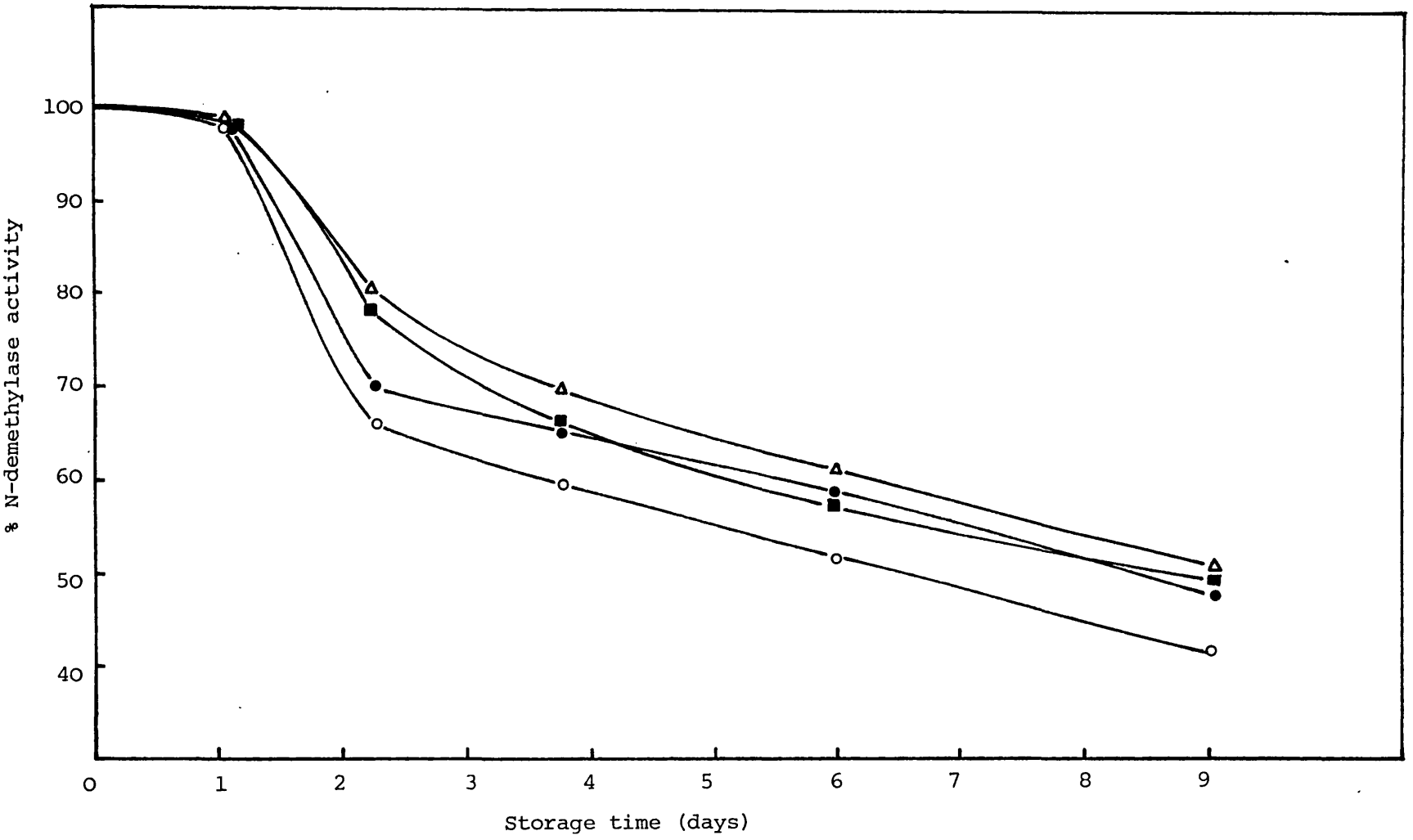


Figure 5.2. The % N-demethylase activity of cell-free extracts of *C. bainieri* with storage time, for extracts stored at 4°C and -20°C in atmospheres of air and nitrogen.

Figure 5.3. The % N-demethylase activity of cell-free extracts of *C. bainieri* with storage time for extracts stored under nitrogen at 4°C in the presence of reducing agents sodium sulphite (▲), L-cystine (●), 2-mercaptoethanol (■) and phosphate buffer control (o).



a 10 day period and the N-demethylase activity after storage compared with an unstored control extract containing no reducing agent.

The % N-demethylase activity with time plotted for the various storage conditions is shown in Figure 5.3. The results showed that there was no improvement in the stability of the cell-free extract by the inclusion of any of the reducing agents investigated. As a result of these stability studies it was decided to store the cell-free extract at $4^{o}C$, in an atmosphere of nitrogen, as 95 - 100% activity would be maintained over the period of time required to conduct any of the experiments described in the following chapters.

5.4.3 Evidence for the presence of cytochrome P-450 in cell-free extracts from *C. bainieri*

Each cell-free extract prepared was subjected to the standard method of Omura and Sato[126] described in Section 5.3.2 to detect the presence of cytochrome P-450. If present, the quantity of cytochrome P-450 was estimated from the UV carbon monoxide difference spectrum assuming that the *Cunninghamella* cytochrome P-450 has the same absorption coefficient as reported by Omura and Sato[126] for $_{liver}$ microsomal cytochrome P-450 (91 cm^{-1} mM^{-1}).

A typical UV spectrum of absorbance against wavelength plotted from 380 nm to 520 nm of a cell-free extract from *C. bainieri* induced with codeine is illustrated in Figure 5.4. A linear baseline

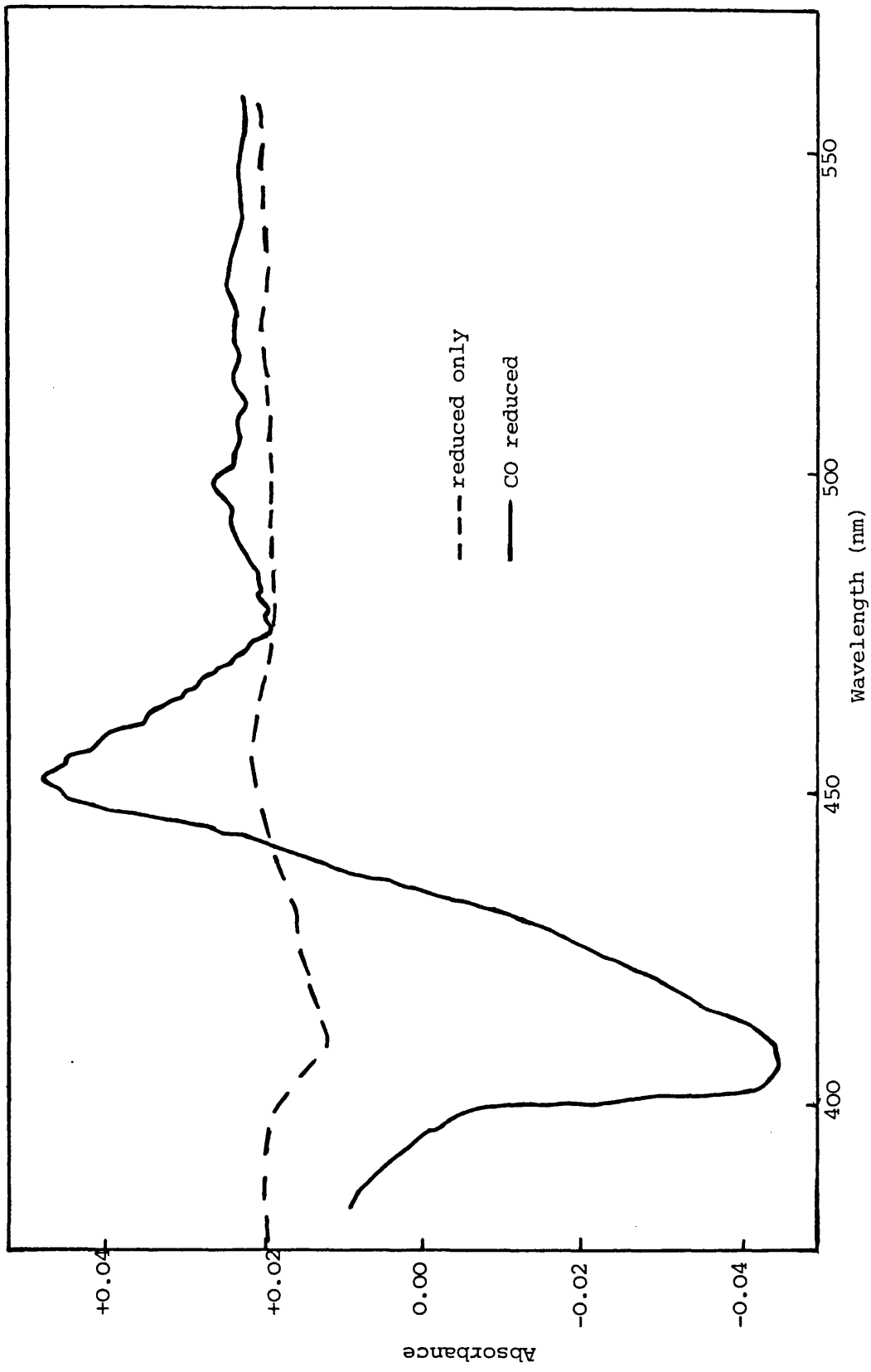


Figure 5.4. UV carbon monoxide difference spectrum of cell-free extract from *C. bainieri* induced with codeine.

was produced from 380 to 520 nm when the cell-free extract, reduced with dithionite, was placed in both sample and reference cuvettes. This is shown by the broken line in Figure 5.4. When carbon monoxide was bubbled for 30 seconds into the extract contained in the sample cuvette only, a maximum absorbance was observed in the 450 nm region of the UV CO reduced difference spectrum, shown by the continuous line in Figure 5.4. This provides strong evidence that the N-demethylase present in *C. bainieri* is a cytochrome P-450 type monooxygenase.

5.4.4 Determination of apparent K_m and V values for the N-demethylation of codeine and codeine N-oxide substrates by cell-free extracts from *C. bainieri*

It has been suggested that the N-oxide could be a possible intermediate in the N-demethylation of tertiary amines by cytochrome P-450 linked monooxygenases[133] (Figure 5.1). The following kinetic experiments were therefore performed to determine the apparent Michaelis constant (K_m) and maximum velocity (V) for the N-demethylation of both codeine and codeine-N-oxide substrates, by cell-free extracts of *C. bainieri*. These results would provide evidence to suggest whether or not the N-oxide is an intermediate in the N-demethylation of codeine by *C. bainieri*.

Incubation mixtures were prepared containing the following:- cell-free extract (1.3 ml), $FeSO_4.7H_2O$ (0.1 mM), NADPH (0.1 mM), NADH (0.1 mM), semicarbazide HCl (3 mM) and 0.066 M phosphate buffer, pH 7.0, to 1.5 mls. Each mixture was incubated at 30°C,

and the reaction initiated by the addition of either codeine, or codeine N-oxide substrates. The experiments were constructed with different substrate concentrations in the range 0.512 to 2.56 mM. The reaction was terminated after 0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 minutes of incubation at 30°C by boiling each mixture for 10 minutes in a water bath. Each mixture was then assayed for norcodeine by the procedure described in Section 4.3.3(b). Plots of the norcodeine concentration (μM) in the incubation mixture against time were constructed, and the initial reaction rates were calculated for each substrate concentration.

These data were used to construct standard Lineweaver-Burke (1/v against 1/ [S]), and Woolf plots ([S]/ v against [S]) shown in Figures 5.5, and 5.6, respectively, where v is the initial reaction rate and [S] is the substrate concentration. From these plots the apparent Michaelis constant (K_M) and $\underline{V}$ values for the N-demethylation of codeine and codeine N-oxide substrates by *C. bainieri* (C_{43}) were determined. The results obtained were:-

Table 5.1. Kinetic data for the N-demethylation of codeine and codeine N-oxide substrates by cell-free extracts of *C. bainieri*.

Substrate	Lineweaver-Burke		Woolf	
	App. $\underline{K_M}$ mM	$\underline{V}$ nm min^{-1}	App. $\underline{K_M}$ mM	$\underline{V}$ nm min^{-1}
Codeine	3.45	0.62	3.97	0.67
Codeine N-oxide	2.56	0.25	3.11	0.28

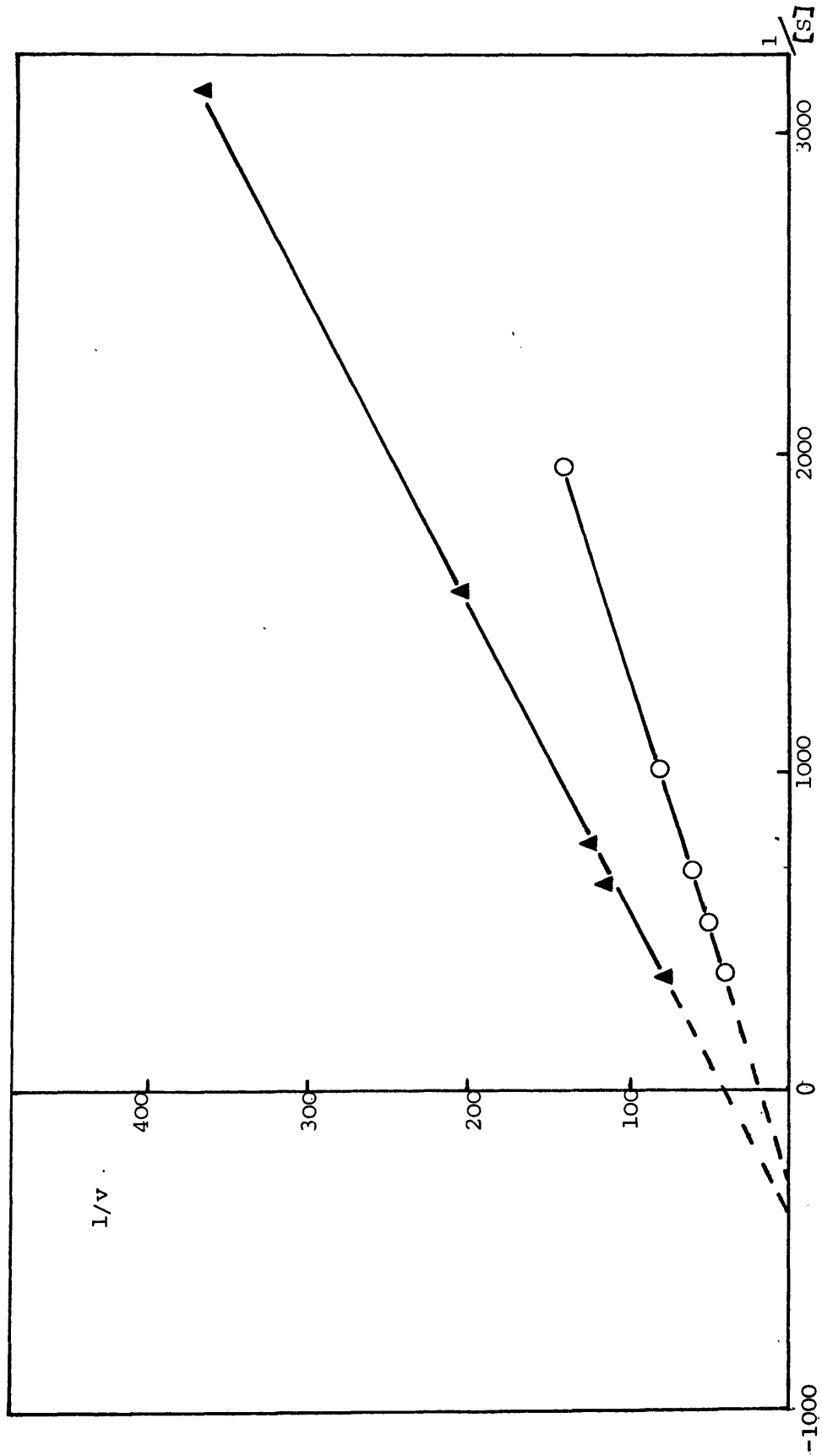


Figure 5.5. Lineweaver-Burke plots of N-demethylation of codeine O and codeine N-oxide ▲ by cell-free extracts from *C. bainieri* at 30°C and pH 7.0

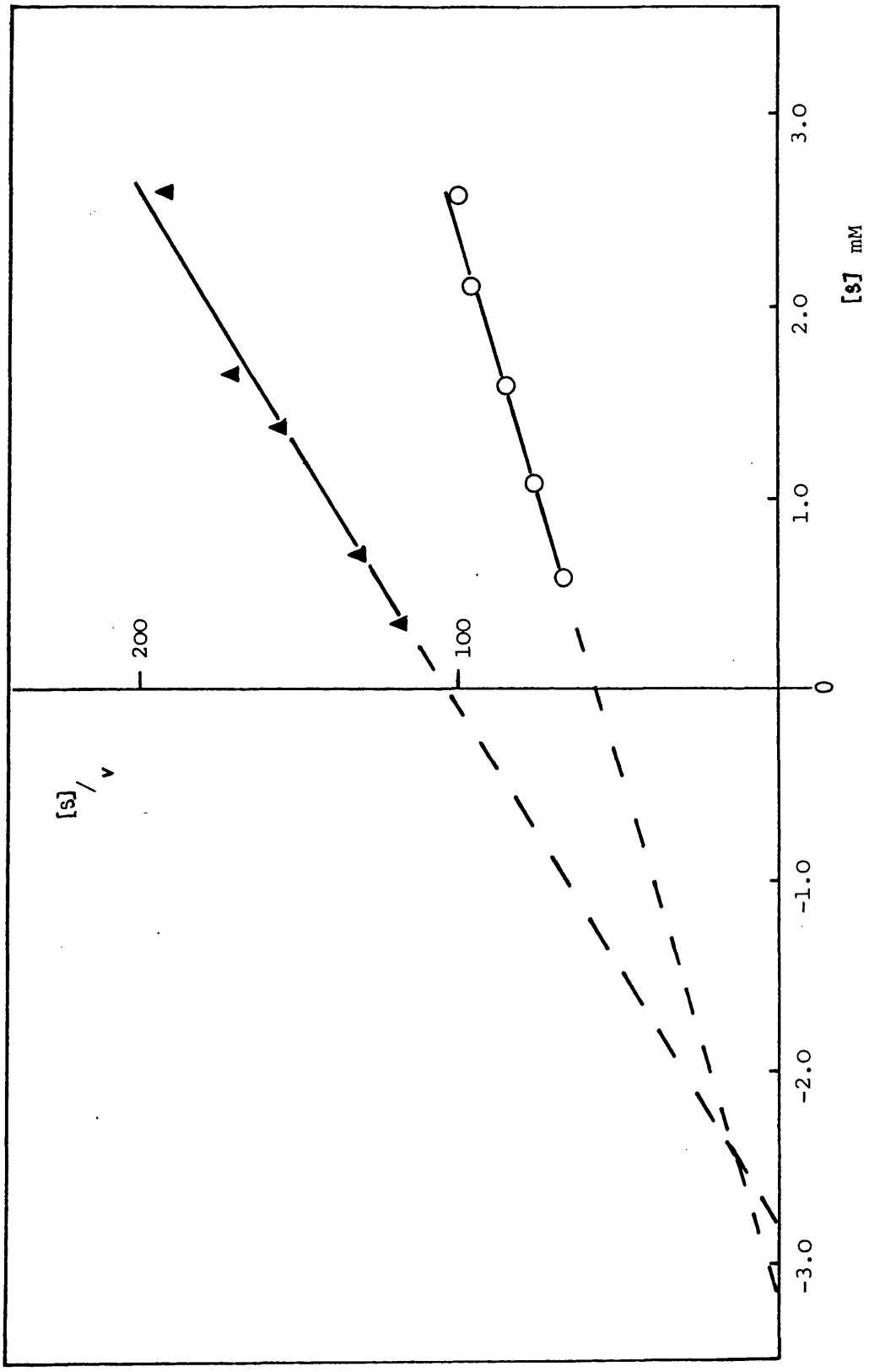


Figure 5.6. Woolf plot of N-demethylation of codeine O and codeine N-oxide ▲ by cell-free extracts from *C. bainieri* at 30°C and pH 7.0

The significance of these results in relation to the mechanism of codeine N-demethylation is discussed on page 223.

5.4.5 The effect of enzyme inducers on codeine N-demethylation by cell-free extracts from *C. bainieri*

Organisms can increase monooxygenase activity to more effectively metabolise certain foreign substances. This response may result in a quantitative enhanced capacity to utilize a given substrate or substrates, and is generally referred to as induction[138]. If codeine N-oxide was an intermediate in the N-demethylation of codeine, it would be expected to be a good inducer of the enzyme.

Enzyme induction experiments were performed by adding various substrates as possible inducers to the medium in which the microorganism, *C.bainieri*, was growing. The effectiveness of each substrate as an inducer was assessed by measuring the specific transformations of cell-free extracts produded from the induced cells. The specific transformation was defined, in this case, as the transformation of codeine to norcodeine per mg of protein in the cell-free extract.

The enzyme inducers listed in Table 5.2 were added to the stage-2 cultures 24 hours after inoculation with the first stage cultures to give a concentration of 1 mM in the medium. Codeine N-oxide, codeine base and codeine phosphate were dissolved in phosphate buffer, pH 7.0, before addition, diazepam was dissolved in 95% alcohol and tetradecane was added directly to the medium to give a final concentration of 1% w/v. Using codeine phosphate

as a test substrate, the N-demethylase activities of the cell-free extracts prepared from induced cultures, were compared to those from cultures containing no inducer . The specific transformations for the conversion of codeine to norcodeine by cell-free extracts prepared from cultures induced with various substrates are given below:

Table 5.2. Effect of inducers on the N-demethylation of codeine by cell-free extracts from *C. bainieri*.

Inducer	*Specific transformation x 10^{-3}
None	0.4
Codeine N-oxide	9.3
Codeine phosphate	14.9
Codeine base	16.2
Diazepam	11.8
Tetradecane	22.6

* specific transformation = μM norcodeine, mM codeine^{-1}, mg^{-1} protein.

The data in Table 5.2 show that codeine N-oxide was found to be a less effective inducer than codeine itself. The significance of these findings are discussed on page 224.

5.4.6 The effect of inhibitors on codeine N-demethylation by cell-free extracts from *C. bainieri*

Cytochrome P-450 monooxygenases from both mammalian[139-140] and microbial[25] systems are known to be inhibited by a variety of substances, including SKF-525A and carbon monoxide[138], whereas potassium cyanide is known to be an N-oxide demethylase inhibitor[141]. In an attempt to characterise the codeine N-demethylase in *C. bainieri*, the effect of these typical inhibitors was investigated. The results of these experiments may then enable a prediction to be made concerning the mechanistic route of codeine N-demethylation by *C. bainieri*.

Incubation mixtures were prepared in glass screw-cap tubes (15 ml), containing the components described in Section 5.4.3, page 210. Duplicate pairs of test and control incubation mixtures (containing no inhibitor), maintained at 4°C, were then subject to one of the following treatments:-

a) untreated - No inhibitor added - control.

b) Potassium cyanide: - Added as a solution in 0.066 M phosphate buffer to provide a concentration of 5 µM.

c) SKF-525A:- - Added as a solution in 0.066 M phosphate buffer to provide a concentration of 1 mM.

d) Carbon monoxide: - Bubbled into the surface of the incubation mixture through a glass pipette for 30 seconds and the tube then closed.

In addition, an incubation mixture was prepared containing cell-free extract which had been boiled at 100°C for 10 minutes.

The incubation mixtures were then equilibriated at 30°C, and the reaction initiated by the addition of codeine or codeine N-oxide substrates respectively in 0.066 M phosphate buffer, pH 7.0, to give a final concentration in each case of 1.5×10^{-3} M, in each test mixture. The tubes were closed, gently shaken and incubated at 30°C for 30 minutes. Samples of each mixture were taken (1 ml), filtered through a 0.45 μm cellulose acetate membrane filter (Millipore), and then assayed for norcodeine by HPLC (see Section 5.3.1). To ensure that the presence of inhibitors would not affect the HPLC assay for norcodeine, the calibration procedure described in Section 2.4.5 was repeated,using standard norcodeine solutions with the addition of inhibitors at the concentrations stated above. In each case it was found that there was no change in the detector response to norcodeine with excellent precision maintained. Data for transformation of codeine to norcodeine by cell-free extracts of *C. bainieri* in the presence of these inhibitors are presented in Table 5.3. A 100% transformation was assumed if no inhibitor was present. The transformations in mixtures containing an inhibitor have been calculated relative to this.

It can be seen from Table 5.3 that the N-demethylation of both codeine and codeine N-oxide by the cell-free extract was inhibited by SKF-525A and carbon monoxide but there was no significant inhibition by potassium cyanide. The significance of these findings is discussed on page 225.

Table 5.3. Effect of enzyme inhibitors on N-demethylation of codeine by cell-free extracts from *C. bainieri* at pH 7.0, 30°C.

INHIBITOR	% RELATIVE TRANSFORMATION	
	CODEINE	CODEINE N-OXIDE
No inhibitor	100	100
Potassium cyanide (5 μM)	96	85
SKF-525A (10^{-3} M)	12	23
Carbon monoxide	9	29
Boiled enzyme	3	4

5.5 Discussion

A cell-free extract which retained its N-demethylase activity over the time required to conduct kinetic experiments was required to facilitate these studies. The % of the initial N-demethylase activity of cell-free extracts from *C. bainieri* remaining with storage time, for extracts stored at $4^{o}C$, - $20^{o}C$, in atmospheres of air, and nitrogen, are illustrated in Figure 5.2. After an initial period of stability over the first 20 hours of storage, the N-demethylase activity of all the extracts decreased between 20 and 70 hours of storage to approximately 60 to 80% of its original activity. The extracts then retained this level of activity over the remaining storage time (about 1 week). The inclusion of reducing agents such as sodium sulphite, L-cystine and 2-mercaptoethanol, on storage at $4^{o}C$ in a nitrogen atmosphere failed to enhance the stability of the cell-free extract (Figure 5.3). However, since the extracts stored at $4^{o}C$, in a nitrogen atmosphere, maintained 95 - 100% of their original activity for approximately 20 hours, this was considered acceptable for subsequent studies. The reason for the loss in activity was uncertain. Ferris *et al.*[25] found that the activity of an aryl hydrocarbon hydrolyase enzyme present in cell-free extracts from *C. bainieri* (ATCC 9244) decreased appreciably on standing at $5^{o}C$, and lost up to 90% of the original activity after 24 hours. Furthermore, they too were unable to stabilise the enzyme by the inclusion of reducing agents, but tests for the presence of protease enzymes in the extract[25] were negative, revealing that the loss of activity was unlikely to be due to endogenous proteolytic enzymes.

In the previous chapter it was established that the N-demethylase enzyme present in the cell-free extracts from *C. bainieri* (C_{43}) was capable of N-demethylating codeine, providing the co-factors NADPH, NADH and iron (Fe^{2+}) were present in the incubation mixture. In this chapter further characterisation of the cell-free extract was carried out by determining the carbon monoxide UV difference spectrum shown in Figure 5.4. A maximum absorbance in the 450 nm region provides strong evidence for the presence of a cytochrome P-450 linked enzyme in the cell-free extract of *C. bainieri* induced with codeine. This is consistent with the findings of Ferris *et al.*[25], who also demonstrated that the hydroxylase enzyme found in cell-free extracts of *C. bainieri* (ATCC 9244) was of a cytochrome P-450 type.

The concentration of cytochrome P-450 found in cell-free extracts of *C. bainieri* prepared by extraction method D was approximately 20 nmol per mg protein, calculated using the absorption coefficients of 91 cm^{-1} mM^{-1} reported by Omura and Sato[126] for liver microsomal cytochrome P-450.

The two proposed mechanisms for the N-demethylation of tertiary amines is illustrated in Figure 5.1. It has been suggested[133] that the N-oxide could be a possible intermediate in the N-demethylation of tertiary amines by cytochrome P-450 linked monooxygenases. If the N-demethylation of codeine was proceeding through an N-oxide the Michaelis constant (K_M) for the transformation of codeine N-oxide would be expected to be smaller than that for the transformation of codeine, because the N-demethylase enzyme would be

expected to have a greater affinity for codeine N-oxide. In addition, the maximum velocity, V for codeine N-oxide transformation should be greater than for codeine transformation, since the codeine to norcodeine reaction would involve two steps if the N-oxide was an intermediate, and would not be expected to proceed as fast as the N-oxide to norcodeine reaction which only involves one step.

Standard Lineweaver-Burke and Woolf plots constructed from the results of kinetic experiments carried out with codeine and codeine N-oxide substrates are shown in Figures 5.5 and 5.6 respectively. Apparent K_m values and V values derived for codeine and codeine N-oxide from these plots are listed in Table 5.1. The K_m values for codeine (3.45 mM; 3.97 mM) and codeine N-oxide (2.56 mM; 3.11 mM) were similar, and the V values observed for codeine were greater than for codeine N-oxide (Table 5.1) suggesting that codeine N-oxide was not an intermediate in the N-demethylation of codeine by *C. bainieri*.

The highly polar character of N-oxides have made difficult a full appreciation of the extent to which N-oxidation of tertiary amines occurs in mammalian systems[133]. In kinetic studies concerned with the oxidation of aniline derivatives by liver microsomes, Kiese[142] found that the K_m value for oxygen utilisation in the N-oxide reaction was 40 times greater than that for the N-demethylation reaction. This seems to rule out N-oxidation as an intermediate step in N-dealkylation, and is consistent with the results described above.

Microbial monooxygenases of the cytochrome P-450 type are known to be inducible for substrates which are added to the medium in which the microorganism is growing[131]. If codeine N-oxide was an intermediate in the N-demethylation of codeine it would be expected to be a good inducer of the enzyme reaction. The results of enzyme induction experiments carried out with *C.bainieri* in Section 5.4.4 are shown in Table 5.2. These results showed that very little transformation occurred if no inducer was used. More important, codeine N-oxide was found to be a less effective inducer than codeine phosphate or codeine base and gave lower specific transformations values for the codeine to norcodeine reaction (Table 5.2). It was even less effective than compounds not chemically related to codeine, such as diazepam and tetradecane. These substances were included in the enzyme induction experiments because it has been demonstrated that *C.bainieri* can N-demethylate diazepam from previous studies[18]. Also, the presence of a cytochrome P-450 containing enzyme system has been demonstrated in yeasts, (e.g. *Candida tropicalis*) grown on tetradecane as the sole carbon source[143], and the results of experiments conducted in chapter 3 of this thesis suggested that tetradecane may be a good inducer of the N-demethylation in *C. bainieri*.

In fact the high specific transformation shown in Table 5.2 suggests tetradecane is a good inducer of the N-demethylase in *C. bainieri*. However, further attempts to characterise the cell-free extracts prepared from tetradecane induced cells from *C. bainieri* was prevented by the turbidity of the resulting extract and interference to the assay procedures.

Further information on the mechanism of microbial N-demethylation was obtained by the use of specific inhibitors. Carbon monoxide and SKF 525A are known as classical inhibitors of cytochrome P-450 monooxygenases in both mammalian[139,140] and microbial systems[25]. Potassium cyanide has been found not to inhibit hepatic cytochrome P-450 or fungal cytochrome P-450[25], but is known to be an N-oxide demethylase inhibitor[141]. Therefore, if the N-demethylation of codeine by *C. bainieri* proceeded via the N-oxide intermediate it would be expected that the reaction would be inhibited by potassium cyanide, but not SKF 525A or carbon monoxide. However, if the transformation was occurring via an alternative intermediate it would be inhibited by SKF 525A and carbon monoxide, only, and would be unaffected by potassium cyanide. The effect of these inhibitors on the transformation of codeine and codeine N-oxide substrates is shown in Table 5.3. When no inhibitor was included in the incubation mixture the transformation, codeine to norcodeine, was assumed to be 100%. The boiled enzyme control showed that norcodeine was produced by enzyme activity and not by chemical breakdown in the test assay. SKF 525A and carbon monoxide were found to inhibit the N-demethylation of both codeine and codeine N-oxide, and provides further evidence that the fungal monooxygenase found in *C. bainieri* was a cytochrome P-450 type. However, potassium cyanide produced no significant inhibition of codeine or codeine N-oxide N-demethylation. In addition, with codeine N-oxide substrate, codeine was found as well as norcodeine in the reaction mixture. These findings suggest that norcodeine was not produced from codeine N-oxide directly, but from the reduction of codeine N-oxide back to codeine, and through a second chemical pathway. The microbial reduction of tertiary amine N-oxides

has been reported for other compounds including pyridine N-oxide[144] and trimethylamine N-oxide[145]. Sindelar *et al.*[146] have investigated the possibility that nicotine-1'-N-oxide was formed as an intermediate in the N-demethylation of nicotine by *M. gypseum* and unexpectedly found the N-oxide was efficiently reduced back to nicotine in cell-free systems[146]. Although the enzyme system involved in N-oxide reduction is not known, an inducible tertiary amine N-oxide reductase has been isolated from *E. coli*[147]. This was found capable of reducing tiaramide N-oxide to tiaramide under anaerobic conditions in the presence of NADPH[135], and strong evidence suggests that this reaction was induced by phenobarbital and 3-methylcholanthrene, while carbon monoxide inhibited the reaction[148]. Similar results were obtained with studies on N,N-dimethylaniline N-oxide reduction[149]. Consequently, it has been proposed[126] that tertiary amine N-oxide reduction and α-carbon oxidation of the resulting teriary amine may be catalysed by the same enzyme. Such a reaction sequence could explain the presence of codeine, as well as norcodeine, in the reaction mixture resulting from the transformation of codeine N-oxide by cell-free extracts of *C. bainieri*. In conclusion, the evidence presented seems to discount the N-oxide as an intermediate in the N-demethylation of codeine by *C. bainieri*, and suggests that N-demethylation proceeds via a different intermediate. This could arise by the attack of molecular oxygen, in the presence of the enzyme, on the carbon alpha to the nitrogen (α-C oxidation) to produce a carbinolamine, N-hydroxymethylcodeine (Figure 5.7). However, the N-hydroxymethylcodeine intermediate was unstable, and hence transient, breaking down to norcodeine and formaldehyde. It has also been proposed[150] that a hydroxymethyl intermediate could arise from the rearrangement of a tertiary

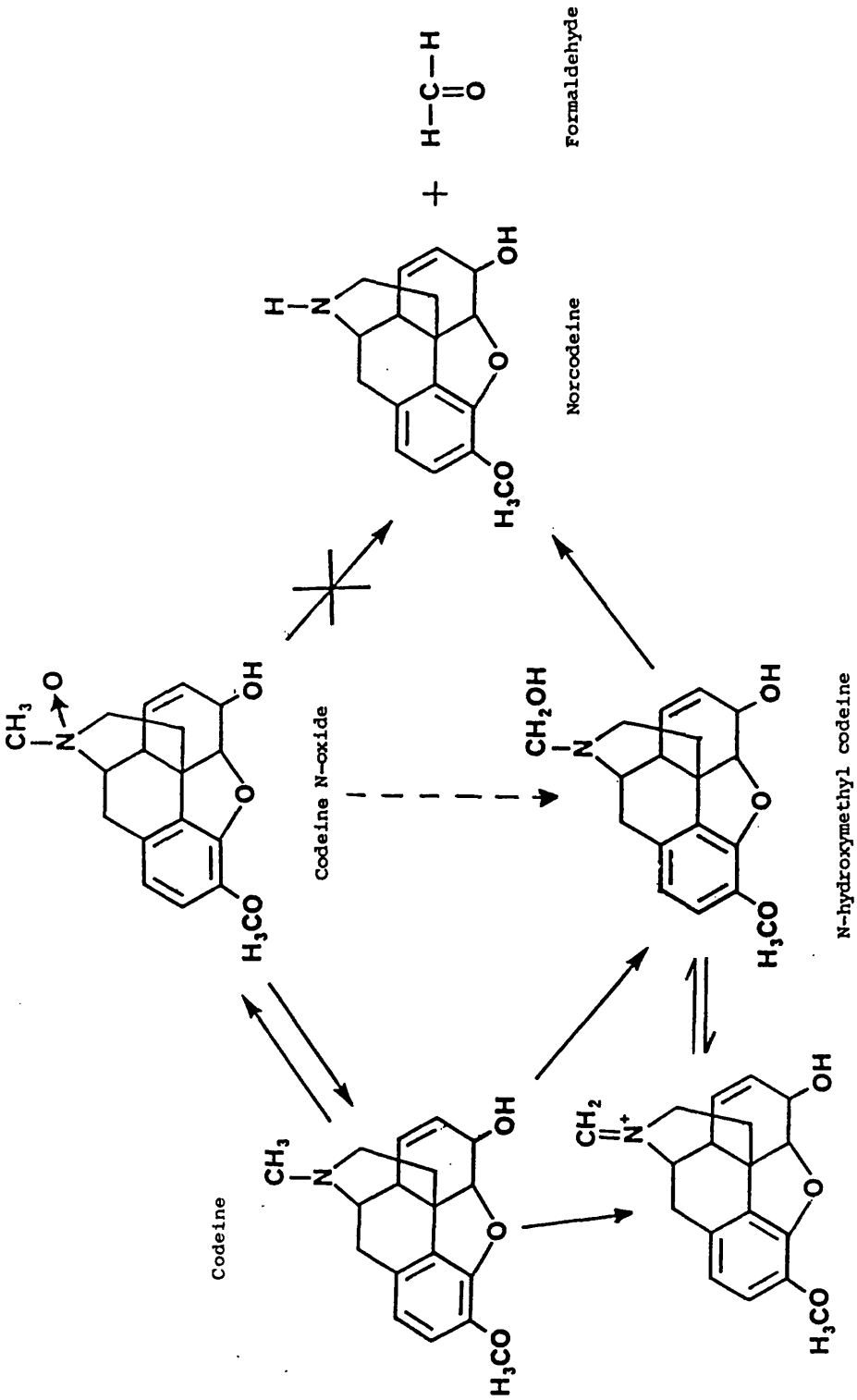


Figure 5.7. Proposed mechanism of N-demethylation of codeine by cell-free extracts of *C. bainieri*

amine N-oxide intermediate. For example, in the N-demethylation of trimethylamine by *Ps. aminovorans*, Large[24] proposed an N-oxide intermediate which was non-oxidatively demethylated to formaldehyde and dimethylamine. However, there is considerable doubt[23] as to whether this mechanism is generally applicable, since other mechanisms must be invoked to explain the dealkylation of compounds other than tertiary amines[22]. Therefore, it seems likely that the rearrangement of codeine N-oxide to N-hydroxymethylcodeine by *C. bainieri* if it occurred, was a minor pathway, since the evidence supports the following two-step reaction pathway leading to the N-dealkylation of codeine N-oxide; (1) reduction of the N-oxide and (2) the cytochrome P-450 mediated N-dealkylation of the resulting tertiary amine, codeine, to norcodeine (Figure 5.7).

The mechanism(s) by which cytochrome P-450 catalysed oxidations proceed is not fully understood. However, the overall stoichiometry of the reaction is believed to involve a two electron oxidation of the substrate by oxygen (O_2) which is coupled to a two electron oxidation of NADPH and the formation of one mole of water. The process is given below with the oxidation of a tertiary amine to the corresponding carbinolamine, and its subsequent decomposition to the secondary amine and formaldehyde[126].

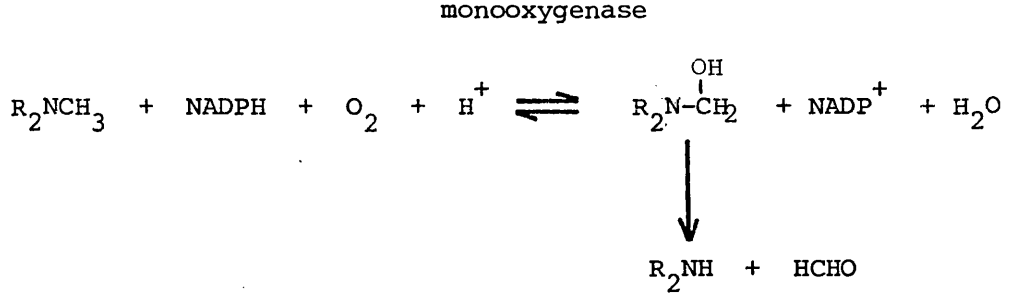
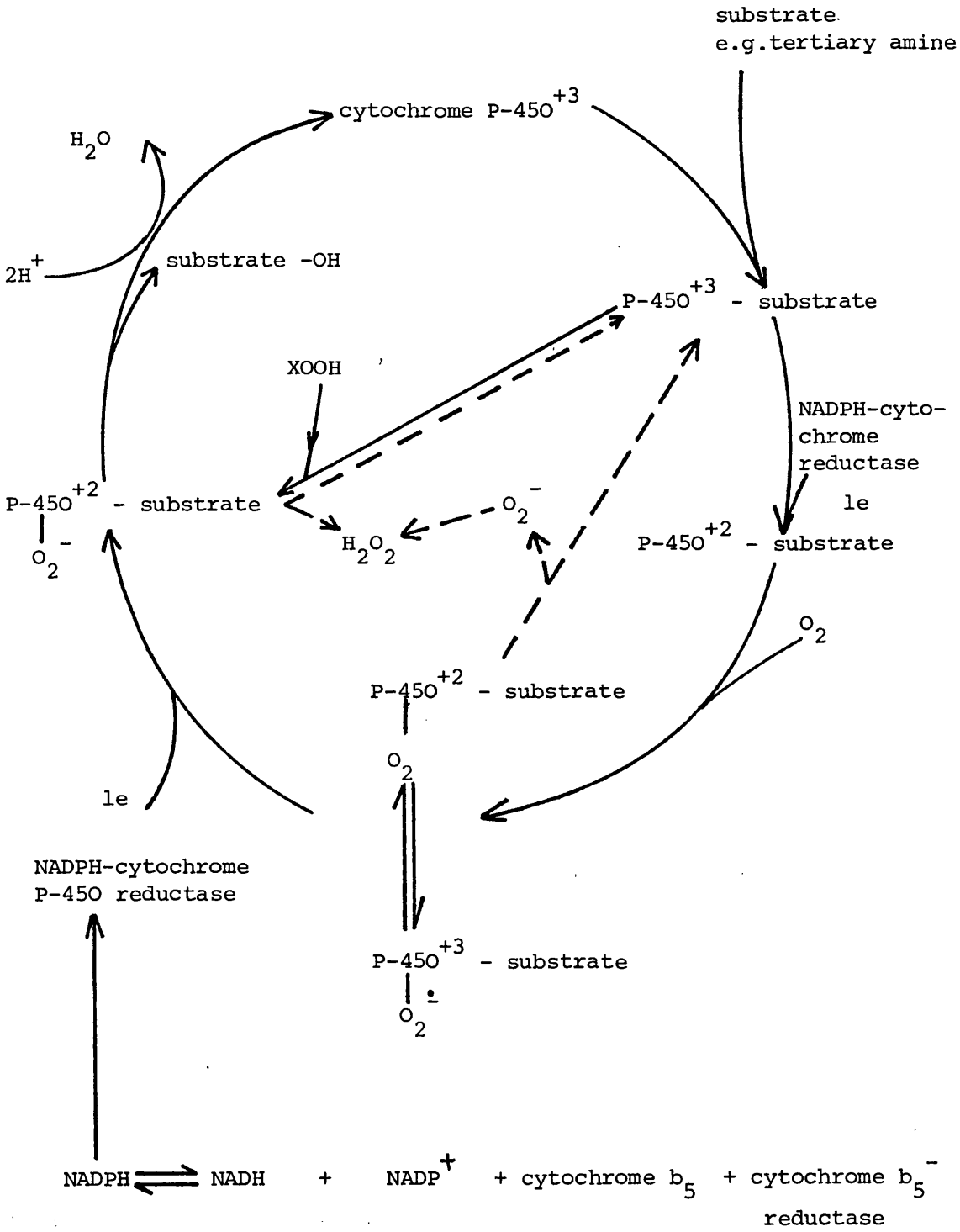


Figure 5.8. Oxidation of substrates via cytochrome P-450 linked monooxygenases



The role of cytochrome P-450 in oxidative transformations is illustrated in Figure 5.8. The process is thought to be initiated by the activation of dioxygen (O_2) to an oxenoid species such as oxene ($:\ddot{O}:$). This electron deficient oxygen atom, is capable of establishing a bond to the tertiary amine substrate at the methyl carbon alpha to the nitrogen atom in the presence of the mono-oxygenase. Present evidence[126] suggests that the substrate (R_2N-CH_3) binds to a hydrophobic region of cytochrome P-450 which is situated in close proximity to the oxidized (Fe^{3+}) iron containing haem group. Once the substrate is bound, the iron is reduced to Fe^{2+} in a reaction catalysed by the NADPH requiring enzyme cytochrome P-450 reductase. This reduced form of cytochrome P-450 accepts dioxygen (O_2) as an axial ligand on the surface to which the substrate is bound (Figure 5.8). This system is reduced by a second electron to form a species which may cleave the oxygen-oxygen bond and yield with the addition of two protons one mole of water and "active oxygen" - namely a ferric-oxene complex ($Fe^{3+}:\ddot{O}:$). However, the details concerning the pathway by which $Fe^{3+}:\ddot{O}:$ converts a tertiary amine to the corresponding α-carbinolamine are not clear. Direct oxygen insertion between the C-H bond has been proposed[151], as well as an alternative pathway which involves an initial electron transfer from nitrogen to oxygen to form bound superoxide radical anion ($Fe^{3+}:\ddot{O}:^{\bullet}$) and the radical cationic nitrogen species ($R_2\overset{+}{N}^{\bullet}-CH_3$). Hydrogen radical abstraction from $R_2\overset{+}{N}^{\bullet}-CH_3$ leads to $Fe^{3+}:\ddot{O}:H$ and the imminium ion $R_2\overset{+}{N}=CH_2$, and final transfer of the hydroxide group to the imminium ion would yield the carbino-lamine product. Although there is some evidence for the involvement

of imminium ion intermediates[126] in the microsomal α-C oxidation of tertiary amines, an alternative pathway involving direct oxene insertion to generate a carbinolamine which might be in equilibrium with the corresponding imminium ion, could also explain these data.

CHAPTER SIX

APPLICATION OF ^{13}C NMR SPECTROSCOPY TO STUDY THE MECHANISM OF CODEINE N-DEMETHYLATION BY CELL-FREE EXTRACTS OF *C. bainieri*.

Chapter 6. Application of ^{13}C NMR spectroscopy to study the mechanism of codeine N-demethylation by cell-free extracts of *C. bainieri*.

6.1 Introduction

In Chapter 5 evidence was presented from kinetic, enzyme induction and enzyme inhibitor studies which seemed to discount the N-oxide as an intermediate in the N-demethylation of codeine by *C. bainieri*. It has been suggested that N-demethylation proceeded via an alternative route involving a carbinolamine intermediate. Unfortunately, this could not be confirmed during those studies since N-hydroxymethylcodeine was unstable, and decomposed to the secondary amine, norcodeine, and formaldehyde.

Some applications of ^{13}C NMR to metabolic and biosynthetic problems were reported in Section 1.8 of this thesis. In this chapter a ^{13}C NMR spectroscopic technique was employed to detect the possible intermediates in the N-demethylation of codeine. Codeine was synthesised with 90% enrichment of the N-methyl group with carbon-13, and this was N-demethylated in the NMR tube by cell-free extracts of *C. bainieri*, so that the intermediates, and subsequently, the formaldehyde liberated, were also ^{13}C enriched. The ^{13}C spectra constructed at progressive times during the enzyme reaction will show signals for the carbon enriched species only, and these could be identified by their chemical shift values and off-resonance splitting patterns, providing further evidence for the reaction mechanism for the N-demethylation of codeine by *C. bainieri*.

6.2 Materials

6.2.1 Chemicals and reagents

Formaldehyde solution (19% w/v) with 90 atom% ^{13}C was purchased from Prochem B.O.C. Ltd., England.

Formic acid (90% w/v), Hydrazine hydrate, phenylhydrazine HCl and sodium metabisulphite were all Analar grade and purchased from B.D.H. Ltd. Poole, England.

Alcohol dehydrogenase, and tetramethylsilane (T.M.S.). - internal standard for NMR, were purchased from Aldrich Chemical Company, Gillingham, Dorset.

6.2.2 Test compounds

Codeine $\left(\text{N-Me-}^{13}\text{C}\right)$; This was prepared in two stages. In stage-one norcodeine was prepared from codeine base (see page 48). In stage-two codeine $\left(\text{N-Me-}^{13}\text{C}\right)$ was prepared from norcodeine by the method of Anderson and Woods[152], employing ^{13}C formaldehyde with 90% atom enrichment.

Norcodeine (1.027 g; 3.6 mmoles), absolute alcohol (42.5 ml), ^{13}C formaldehyde (0.67 ml; 4.25 mmoles) and formic acid 90% w/v (4.25 ml; 80 mmoles) were heated under gentle reflux for 90 minutes in a 50 ml. round bottomed flask. The alcohol and excess formic acid were removed by rota evaporation under reduced pressure leaving an oily residue. This was dissolved in HCl (0.25 N, 17.5 ml.), and the crude product precipitated by addition of N. NaOH to pH 9. Chloroform (3 x 20 ml.) was used to extract the product.

The chloroform layer was washed with water (25 ml.), dried (anhydrous $MgSO_4$) and evaporated to yield a white powder (1.028 g). This was dissolved in the minimum of hot ethanol, and ethanolic HCl added dropwise until the solution was acidic. Cooling to room temperature yielded white crystals (1.07 g) of codeine $\left(\text{N-Me-}^{13}\text{C}\right)$ HCl.

Analytical;

melting point, 279°C (lit.[78] 280°C).

^{1}H NMR, H^{δ} ($CDCl_3$ codeine $\left(\text{N-Me-}^{13}\text{C}\right)$ base); 1.86 (2H, m, C_{15}-H); 2.44 (5H, m, C_{16}-H, C_{10}-H, C_{14}-H); 2.32 (3H, s, N-Me); 3.29 (1H, m, C_9-H); 3.73 (3H, s, O-Me); 3.90 (1H, s, O-H, exchanges with D_2O); 4.21 (1H, m, C_6-H); 4.84 (1H, d, C_5-H); 5.29 (1H, m, C_7-H); 5.72 (1H, d, C_8-H); 6.6 (2H, d.d, C_1-H, C_2-H)

^{13}C NMR (D_2O; codeine $\left(\text{N-Me-}^{13}\text{C}\right)$ HCl; 21.54(OFR, t, C-10); 36.3 (OFR t; C-15); 38.7 (OFR d; C-9); 41.5 (OFR q; C-17 N-Methyl); 42.2 (OFR s; C-13); 47.4 (OFR t; C-16); 56.7 (OFR q, C-18); 60.8 (OFR d, C-14); 66.4 (OFR d; C-6); 91.4 (OFR d, C-5); 114.6 (OFR d, C-1); 120.8 (OFR d, C-2); 124.65 (OFR s, C-11); 126.2 (OFR d, C-8); 129.6 (OFR s, C-12); 133.96 (OFR d, C-7); 142.46 (OFR s, C-3); 147.0 (OFR s, C-4).

Codeine N-oxide was prepared from codeine base by the method described in Section 5.2.2 (see page 204).

^{13}C NMR $^{\delta}$C (D_2O; codeine N-oxide); 26.0 (OFR t, C-10); 30.9 (OFR t; C-15); 34.0 (OFR d, C-9); 41.9 (OFR s, C-13) 57.0 (OFR t, C-16); 58.0 (OFR q; C-17 N-methyl); 58.3(OFR q, C-18 O-Methyl);

59.6 (OFR d; C-14); 66.7 (OFR d; C-6); 74.7 (OFR d; C-5); 114.95 (OFR d; C-1); 120.85 (OFR d; C-2); 124.5 (OFR s; C-11); 127.5 (OFR d; C-8); 130.1 (OFR s; C-12); 133.1 (OFR d; C-7); 142.6 (OFR s; C-3); 147.0 (OFR s; C-4).

6.2.3 Equipment and instrumentation

NMR spectrometer: A Jeol FX 90Q high resolution Fourier Transform NMR spectrometer operating at 22.5 MHz. was used to observe ^{13}C resonances. This was interfaced to a FA-100 NMR data system based on the 980 B computer with 8 K data points. For an average spectral width of 5000 Hz, a 4 μsecond pulse, corresponding to a tilt angle of 30° was employed, with a 1.8192 second interval (acquisition time + 1 second pulse delay) between pulses. Probe temperature control; The NMR spectrometer was installed in an air conditioned room maintained at a temperature of $21 \pm 0.5^{\circ}C$. A Jeol JES-VT temperature controller was employed to vary the probe temperature from 5 - $40^{\circ}C$ ($\pm 0.5^{\circ}C$). For low temperature control (below $21^{\circ}C$) nitrogen gas evaporated from liquid nitrogen in a 10 litre metal Dewar flask was employed, the evaporation rate was controlled automatically. For high temperature control (above $21^{\circ}C$) air from an air compressor passed through the heat blasting pipe was employed, the temperature of the air was automatically controlled.

NMR sample tubes; glass, 10 mm internal diameter. Tubes were prepared as described in Chapter 4, page 157 . A sealed D_2O capillary was used to provide the lock signal.

6.3 Methods

6.3.1 General methods

Shake flasks were prepared and inoculated with *C. bainieri* according to the procedures and incubation conditions described in Section 3.3.1. Codeine phosphate was added after 24 hours incubation of the stage-two cultures to give a codeine concentration of 1 mM. After 10 days the microbial cells were harvested by filtration, washed with ice-cold 0.066 M phosphate buffer, pH 7.0 and extracted according to Method D, Section 4.3.2 to produce the cell-free extract used in thse studies. Protein in the cell-free extract was determined by the method reported in Section 4.3.2.

6.3.2 NMR procedure

The probe temperature was adjusted to the required value using the Jeol JES-VT temperature controller, and the instrument was allowed to stabilise at this temperature. The sample in the NMR tube was lowered into the NMR probe and spun by compressed air at an average rate of 30 r.p.m. The instrument was then locked onto the ^{2}H signal of the deuterated solvent. Spectra were initially constructed in the NMR mode of operation (complete decoupled spectra), and then the operation repeated in the OFR mode (off-frequency) to observe ^{1}H-^{13}C coupling.

6.4 Experimental

6.4.1 NMR of standard solutions

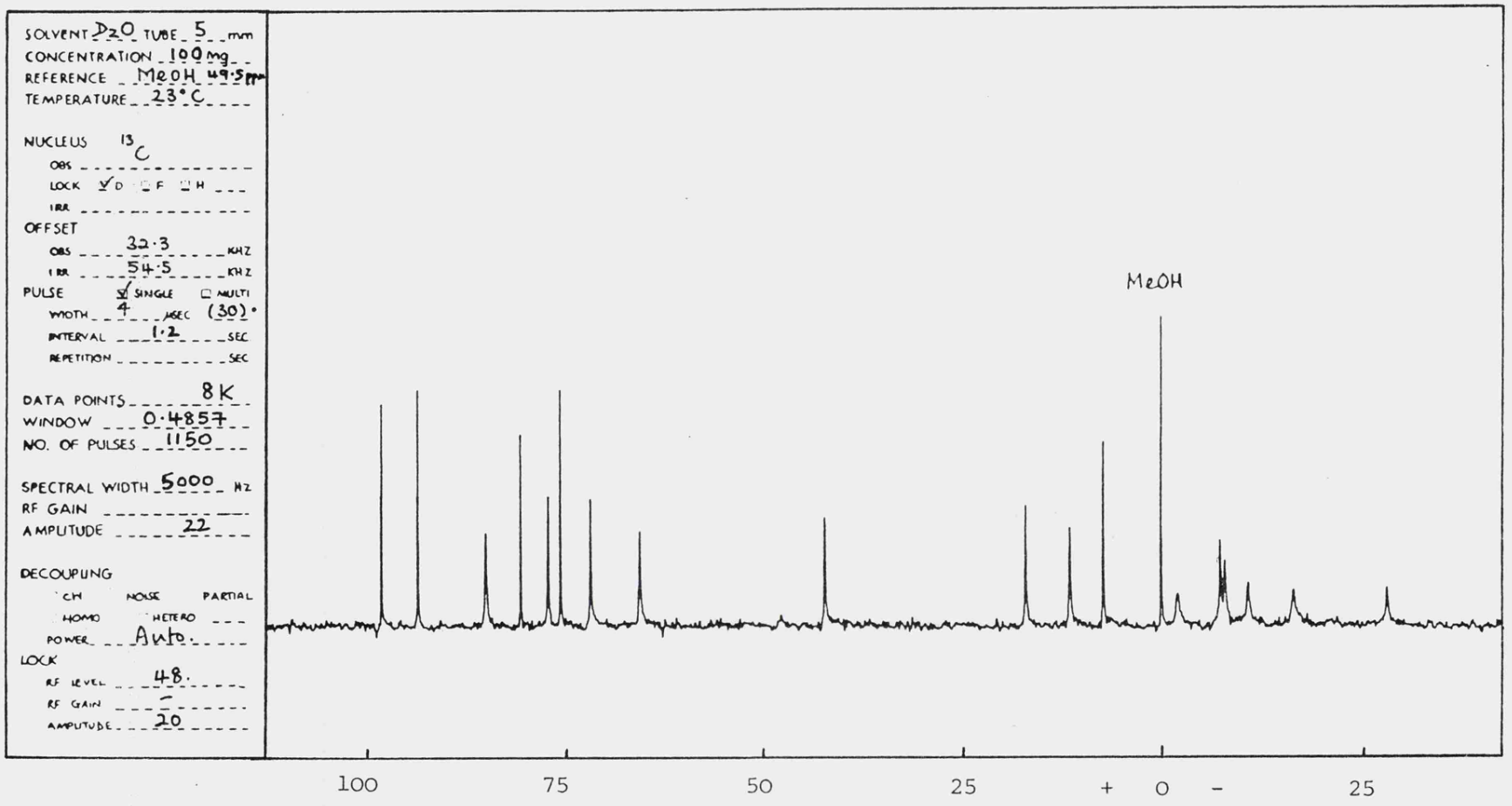
^{13}C NMR spectroscopy was performed on concentrated solutions of codeine hydrochloride and codeine N-oxide (100 mg in 0.5 ml) prepared in D_2O. The complete decoupled spectra (C.O.M.) and off-resonance spectra (OFR) constructed for each solution are illustrated in Figures 6.1 to 6.4. The chemical shifts were calculated with reference to methanol (MeOH 49.5 ppm).

NMR spectra were interpreted with the aid of reference compounds and chemical shift values assigned to the N-methyl carbons of codeine HCl and codeine N-oxide. These were 41.5 ppm and 58.3 ppm respectively, with TMS as a reference (0 ppm).

6.4.2 NMR of formaldehyde and various 'trapping agents'

Microbial N-demethylation of codeine produces norcodeine and an equimolar quantity of formaldehyde. Free formaldehyde is capable of combining with other components in the transformation mixture to produce various ^{13}C labelled artefacts. For example,it could under certain conditions react with norcodeine to form a geminal diamine[18]. To prevent this occurring, formaldehyde may be 'trapped' by the addition of a reagent to the reaction mixture with which it will react to give a single product. The trapping agents most commonly used are carbonyl scavenger compounds which have the ability to react rapidly and quantitatively with compounds

Figure 6.1. COM NMR spectrum of codeine hydrochloride in D_2O



Chemical shift (ppm from MeOH)

Figure 6.2. OFR NMR spectrum of codeine hydrochloride in D_2O

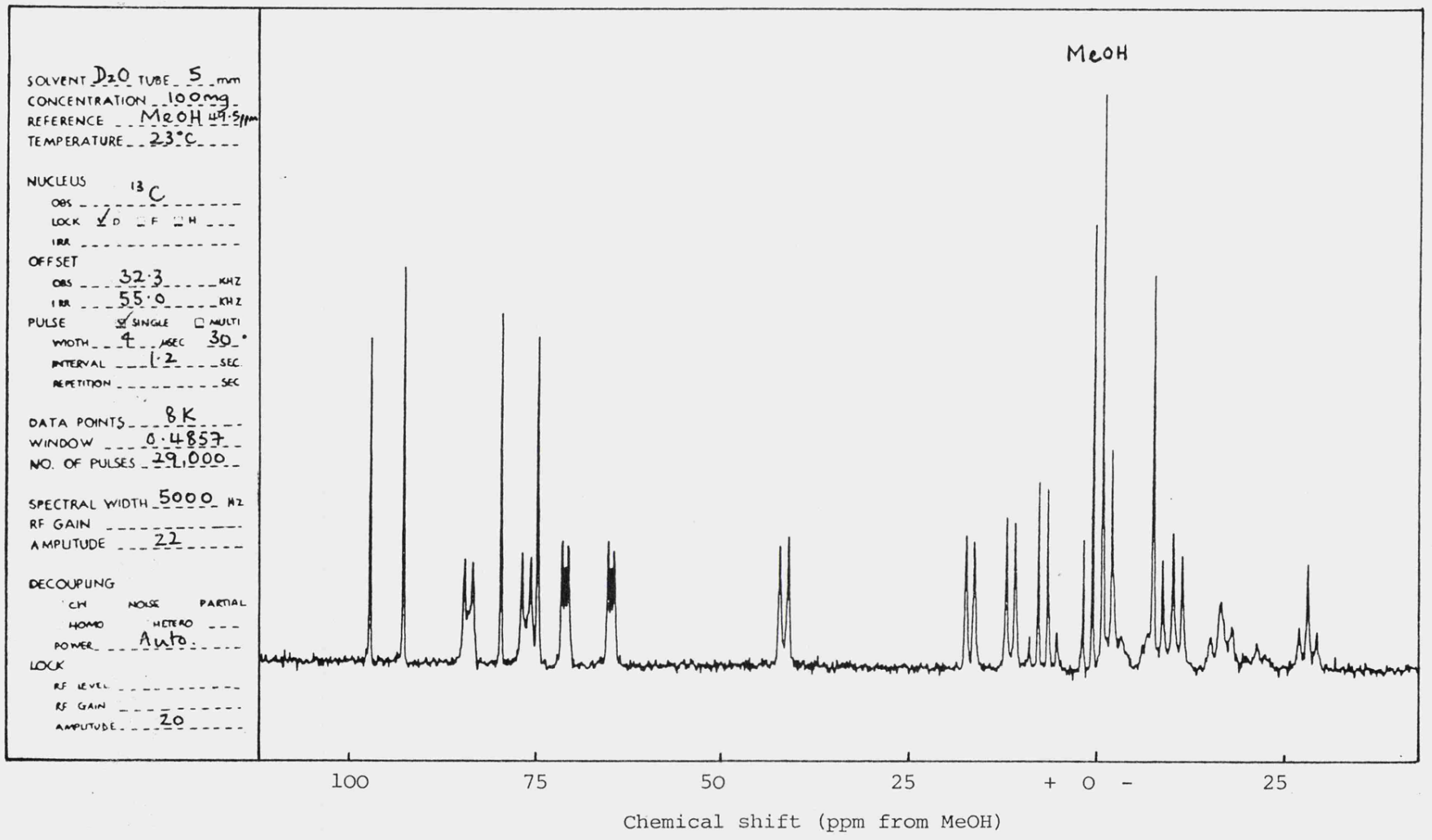


Figure 6.3. COM NMR spectrum of codeine-N-oxide in D_2O

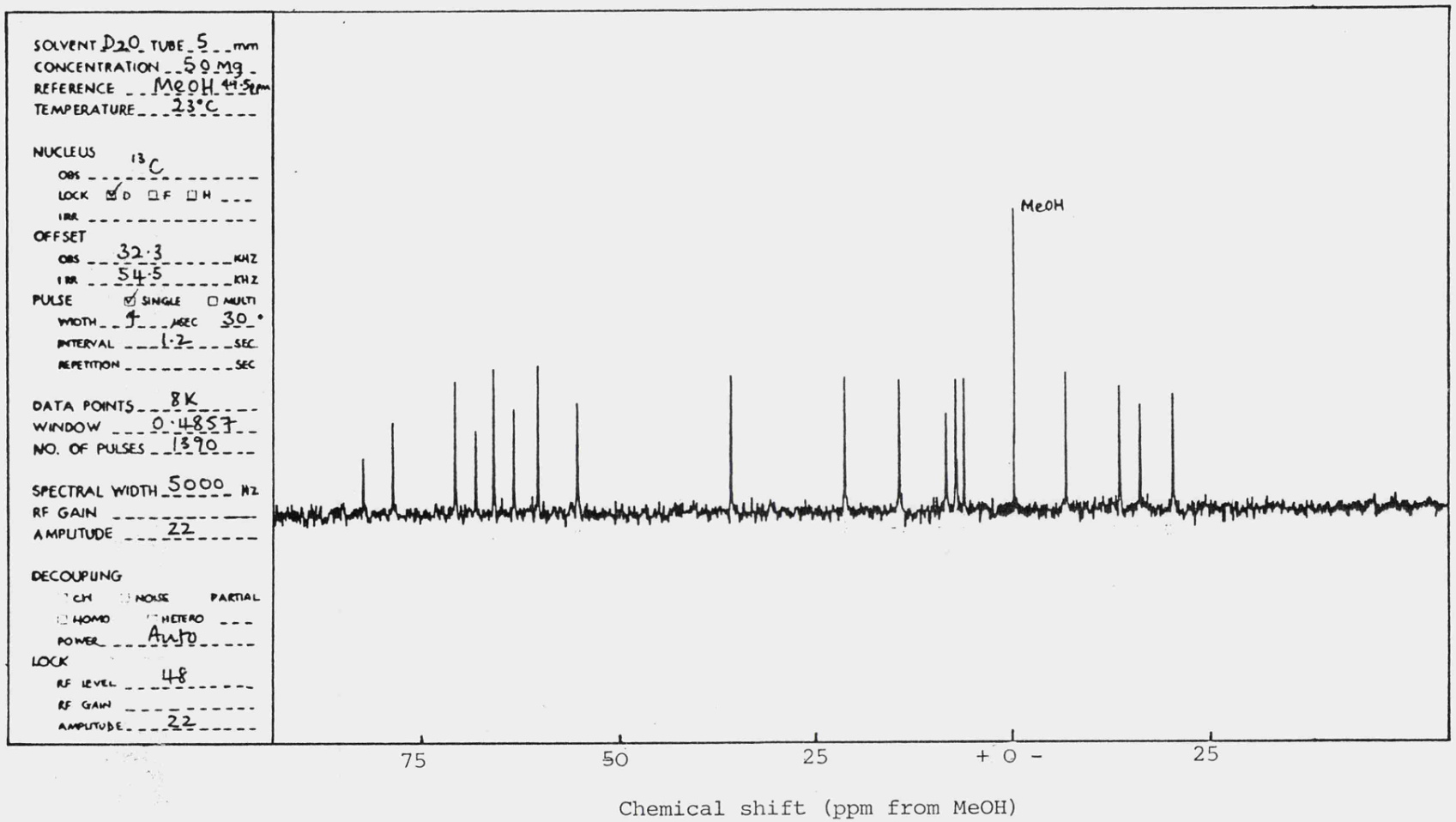
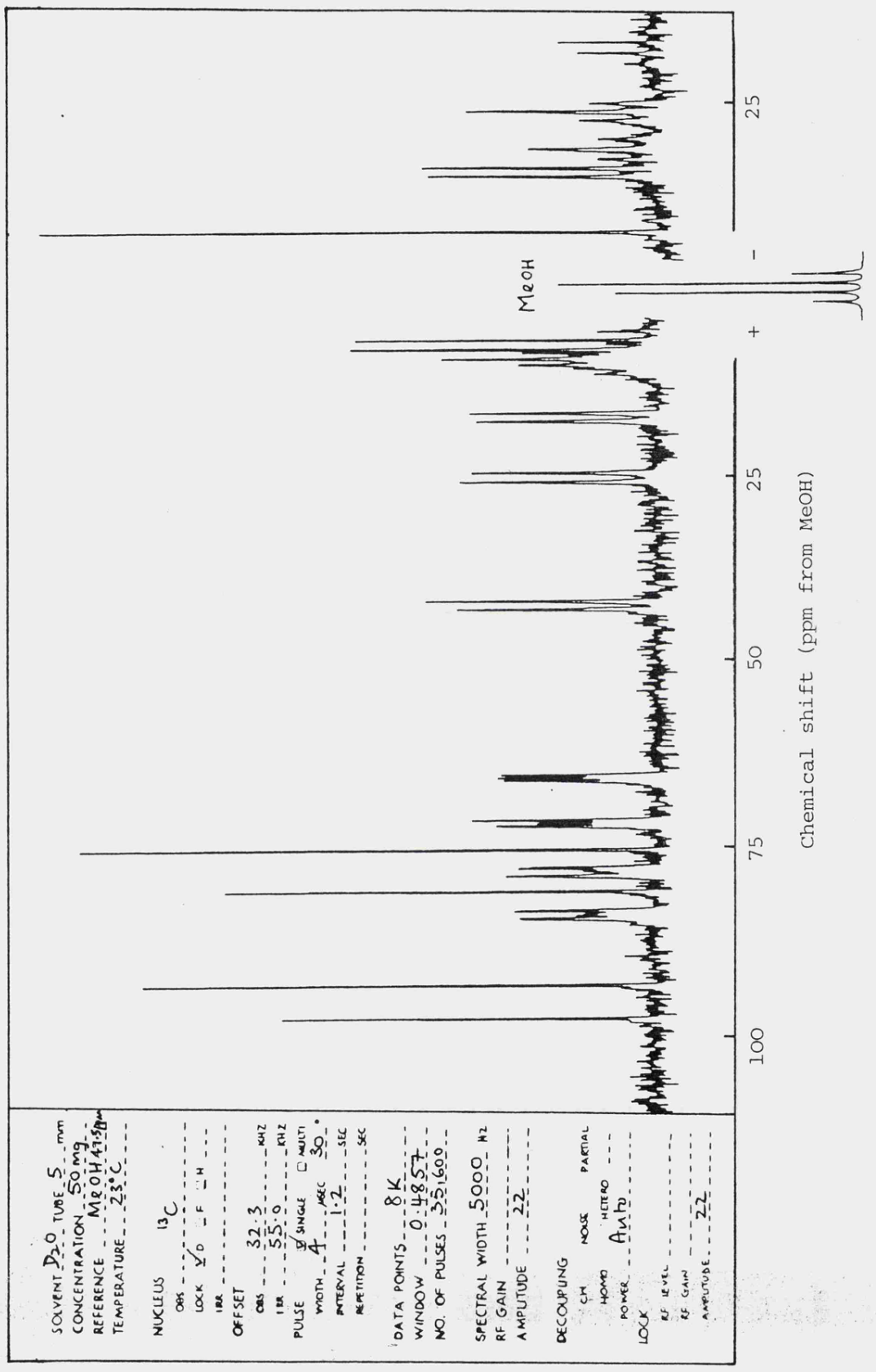


Figure 6.4. OFR NMR spectrum of codeine N-oxide in D_2O



such as formaldehyde, to form non-volatile adducts.[153].

^{13}C NMR spectroscopy was performed on solutions in D_2O (3 mM) of each of the trapping agents listed in Table 6.1 in the presence of ^{13}C labelled formaldehyde solution (50 µl). The sodium bisulphite solution was prepared freshly from sodium metabisulphite solution (5% w/v) to give a final concentration with formaldehyde and D_2O of 1% w/v sodium bisulphite. Alcohol dehydrogenase (2 I.U.) and NADH (10^{-4} M) were added to the formaldehyde solution (50 µl) in 0.5 ml. D_2O.

Chemical shift values for ^{13}C NMR spectra determined for ^{13}C labelled formaldehyde with various trapping agents are shown in Table 6.1. The chemical shift values for the N-methyl carbons of codeine HCl and codeine N-oxide have also been included in Table 6.1 for comparison.

Ideally, the trapping agent employed in NMR enzyme transformation studies was required to possess one signal in the complete decoupled ^{13}C NMR spectrum (COM) and be well displaced from codeine $\left(N\text{-}Me\text{-}^{13}C\right)$HCl and possible intermediates, in the N-demethylation reaction. This was in order to simplify interpretation of the resulting spectra. All the trapping agents investigated possessed one signal only, except for semicarbazide and hydrazine (Table 6.1).

Table 6.1 Chemical shifts of various trapping agents with formaldehyde

Substance	Chemical nature of species	Chemical shift from TMS of ^{13}C-enriched species (ppm)
Codeine $[N\text{-}Me\text{-}^{13}C]HCl$	$[R_2\text{-}N\text{-}\overset{*}{C}H_3]Cl^-$ (H$^+$)	41.6
Codeine N-oxide	$R_2\text{-}N(\rightarrow O)\text{-}\overset{*}{C}H_3$	58.3
Semicarbazide HCl + $\overset{*}{H}CHO$	$NH_2CONH\text{-}N{=}\overset{*}{C}H_2$	56.9,59.9
Hydrazine hydrate + $\overset{*}{H}CHO$	$NH_2\text{-}N{=}\overset{*}{C}H_2$	137.8,75.9,70.8
Phenylhydrazine HCl + $\overset{*}{H}CHO$	$PhNH\text{-}N{=}\overset{*}{C}H_2$	82.4
1% sodium bisulphite + $\overset{*}{H}CHO$	$\overset{*}{C}H_2(OH)SO_3^-Na^+$	74.7
Sodium sulphite + $\overset{*}{H}CHO$	$\overset{*}{C}H_2(ONa)SO_3^-Na^+$	79.4
Alcohol dehydrogenase NADH + $\overset{*}{H}CHO$	$\overset{*}{C}H_3\text{-}OH$	49.5

Notes: 1) The enriched carbon is shown by an asterisk (*)

6.4.3 N-demethylase activity of cell-free extract in the presence of trapping agents

At the concentrations used the trapping agents were required not to interfere with the N-demethylation process. This was investigated by determining the transformation activity of the cell-free extract in the presence of each trapping agent in the following incubation mixtures:- cell-free extract (1.3 ml), $FeSO_4.7H_2O$ (0.1 mM), NADH (0.1 mM), NADPH (0.1 mM) and one of each of the trapping agents:- semicarbazide HCl (3 mM), phenylhydrazine HCl (3 mM), sodium bisulphite (1% w/V), sodium sulphite (3 mM) or alcohol dehydrogenase (2 I.U.)/NADH (0.1 mM) in 0.066 M phosphate buffer (0.2 ml.) pH 7.0. The mixtures were incubated at 30 °C, and the reaction was initiated by the addition of codeine phosphate (3.0 mM) as substrate. After 30 minutes the reaction was terminated by boiling the mixtures for 10 minutes at 100°C. Each of the mixtures was centrifuged at 10,000 r.p.m. for 5 minutes, and the supernatant filtered through a 0.45 μm Millipore type HA filter, prior to the determination of norcodeine by the HPLC method described in Section 2.4.5. The results obtained are shown in Table 6.2.

It was shown in previous studies[18] that semicarbazide HCl does not impair the N-demethylation process, and so the activity of the cell-free extract in the presence of semicarbazide HCl. was assumed to be 100% (Table 6.2). The activity of the cell-free extract in the presence of the other trapping agents is shown in Table 6.2, in relation to the semicarbazide system, and showed that only phenylhydrazine substantially interfered with the N-demethylation process. However, for reasons discussed later

Table 6.2. Transformation of codeine to norcodeine by cell-free extracts of *C. bainieri* in the presence of various formaldehyde trapping agents

Formaldehyde trapping agents	% Transformation to norcodeine (relative to semicarbazide)
Semicarbazide HCl	100
Phenyl hydrazine HCl	18
Sodium bisulphite	95
Sodium sulphite	95
Alcohol dehydrogenase NADH	97

(page 256) sodium sulphite was preferred as the formaldehyde trapping agent, and was employed in subsequent NMR enzyme transformation studies described in this chapter.

6.4.4 ^{13}C NMR chemical shifts of N-methylcarbons in the presence of the test mixture components

It was possible that the test mixture components may affect the NMR chemical shift values of the N-methyl carbons of codeine $\left(N\text{-}^{13}CH_3\right)$ HCl and the sulphite-formaldehyde adduct. Therefore these values were determined, at the operating temperature of the instrument ($23^{o}C$), employing the following test mixture:- boiled cell-free extract (1.3 ml), $FeSO_4.7H_2O$ (0.1 mM), NADH (0.1 mM), NADPH (0.1 mM), sodium sulphite (3 mM), ^{13}C labelled formaldehyde solution (50 µl) and codeine $\left(N\text{-}Me\text{-}^{13}C\right)$HCl (10 mg in 0.066 M phosphate buffer, pH 7.0, 0.2 ml). TMS was added as a reference standard.

The chemical shift value for the N-methyl carbon of codeine $\left(N\text{-}Me\text{-}^{13}C\right)$HCl was observed to be 41.6 ppm with reference to TMS (0 ppm), and was not significantly different to the value of 41.5 ppm obtained in the absence of test mixture components (page 237). In the enzyme transformation mixtures no internal reference was used, so the chemical shift of the formaldehyde-sulphite adduct was determined relative to codeine $\left(N\text{-}Me\text{-}^{13}C\right)$ HCl. This was observed to be + 37.2 ppm with reference to codeine or, 78.8 ppm with reference to TMS, in the test mixture investigated at $23^{o}C$.

6.4.5 ^{13}C NMR spectroscopy of enzyme transformation mixture at 23°C

The NMR enzyme transformation experiment was initially performed at the normal operating temperatures of the instrument (23°C). The enzyme reaction was initiated by adding codeine $\left(N\text{-}Me\text{-}^{13}C\right)HCl$. (10 mg) in a solution (0.2 ml) of $FeSO_4.7H_2O$ (0.1 mM). NADH (0.1 mM), NADPH (0.1 mM) and sodium sulphite (3 mM) in phosphate buffer (0.066 M, pH7.0), to cell-free extract (1. 8 ml) contained in a 10 mm. i.d. NMR sample tube. The NMR instrument was locked onto the D_2O capillary placed coaxially inside the NMR tube , and accumulation of spectra commenced in the COM mode. ^{13}C NMR spectra were constructed at frequent intervals to observe the progression of the enzyme transformation. The operation was repeated in the OFR mode with a fresh enzyme transformation mixture, as described above. A typical series of COM NMR spectra produced at progressive time intervals is shown in Figure 6.5. A signal corresponding to the N-methyl group of codeine $\left(N\text{-}Me\text{-}^{13}C\right)$ HCl (A) was seen above the noise level after only 6 pulses. A signal (B) at + 37 ppm, relative to codeine, was observed after 250 pulses. This corresponded to the formaldehyde-sulphite adduct, and showed that the N-methyl group had been completely cleaved. Resonances due to intermediates in the N-demethylation reaction would occur downfield from codeine if the N-methyl group was oxidised by the N-demethylase enzyme in the cell-free extract. The signal (C) in Figure 6.5 at + 26 ppm, relative to codeine, was clearly seen at 500 pulses corresponding to a possible intermediate.

Figure 6.5. Time-dependent ^{13}C COM NMR spectra for the N-demethylation of (N-Me-^{13}C) codeine by cell-free extract of *Cunninghamella bainieri*. (Temp. 23°C, pH 7.0)
a) 6 pulses; (b) 250 pulses; (c) 500 pulses; (d) 1000 pulses, e) 1500 pulses. A. codeine N-CH_3; B. Intermediate; C sulphite-adduct HO-CH_2SO_3Na

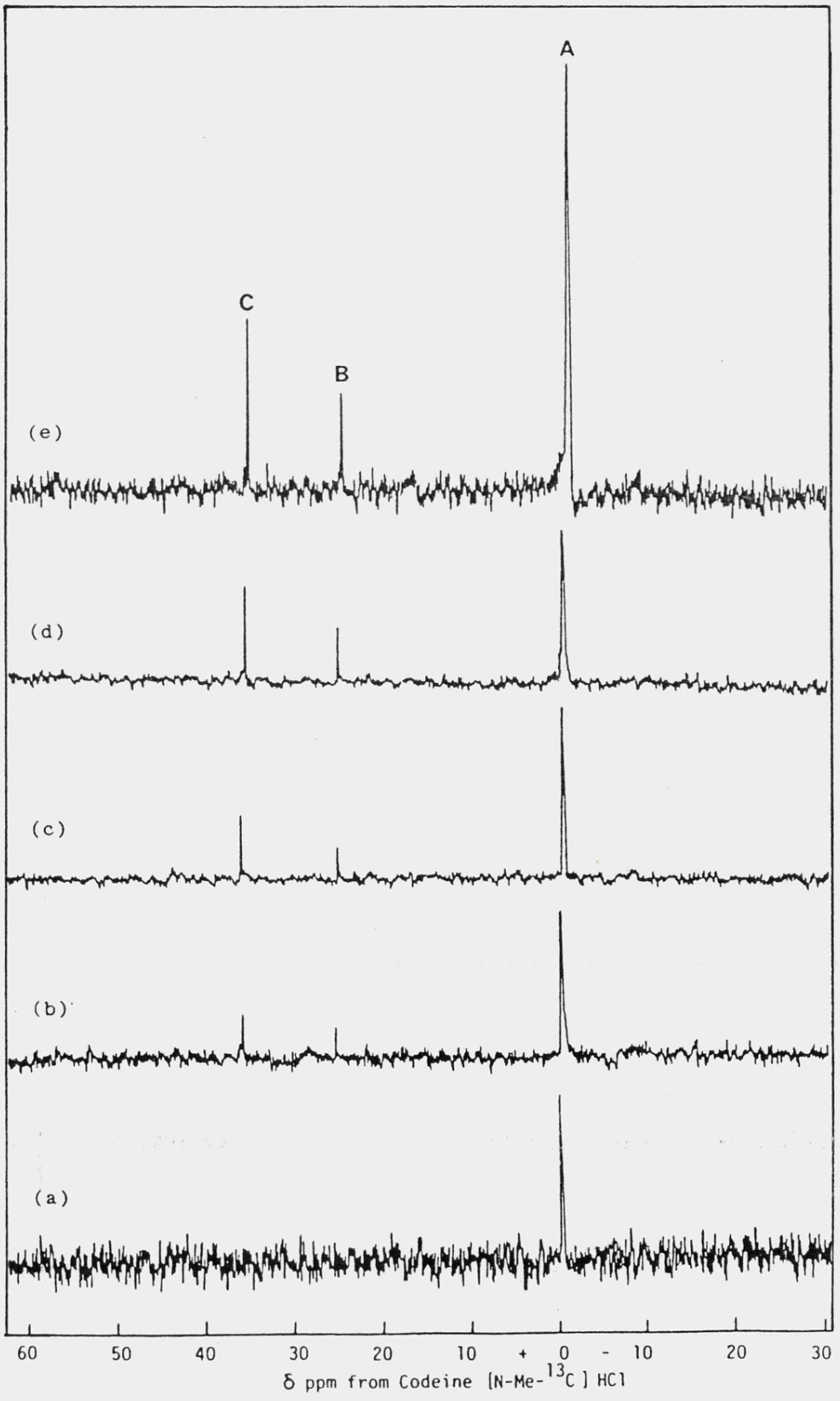


Figure 6.6. ^{13}C NMR OFR spectrum for the N-demethylation of (N-Me-^{13}C) codeine by cell-free extract of *Cunninghamella bainieri* (Temp.$_{*}^{23}$C, pH 7.0, 28,000 pulses). A. Codeine N-CH_3, quartet; B. Intermediate, triplet; C. Sulphite-adduct HO-$\overset{*}{C}H_2$-SO_3Na, triplet.

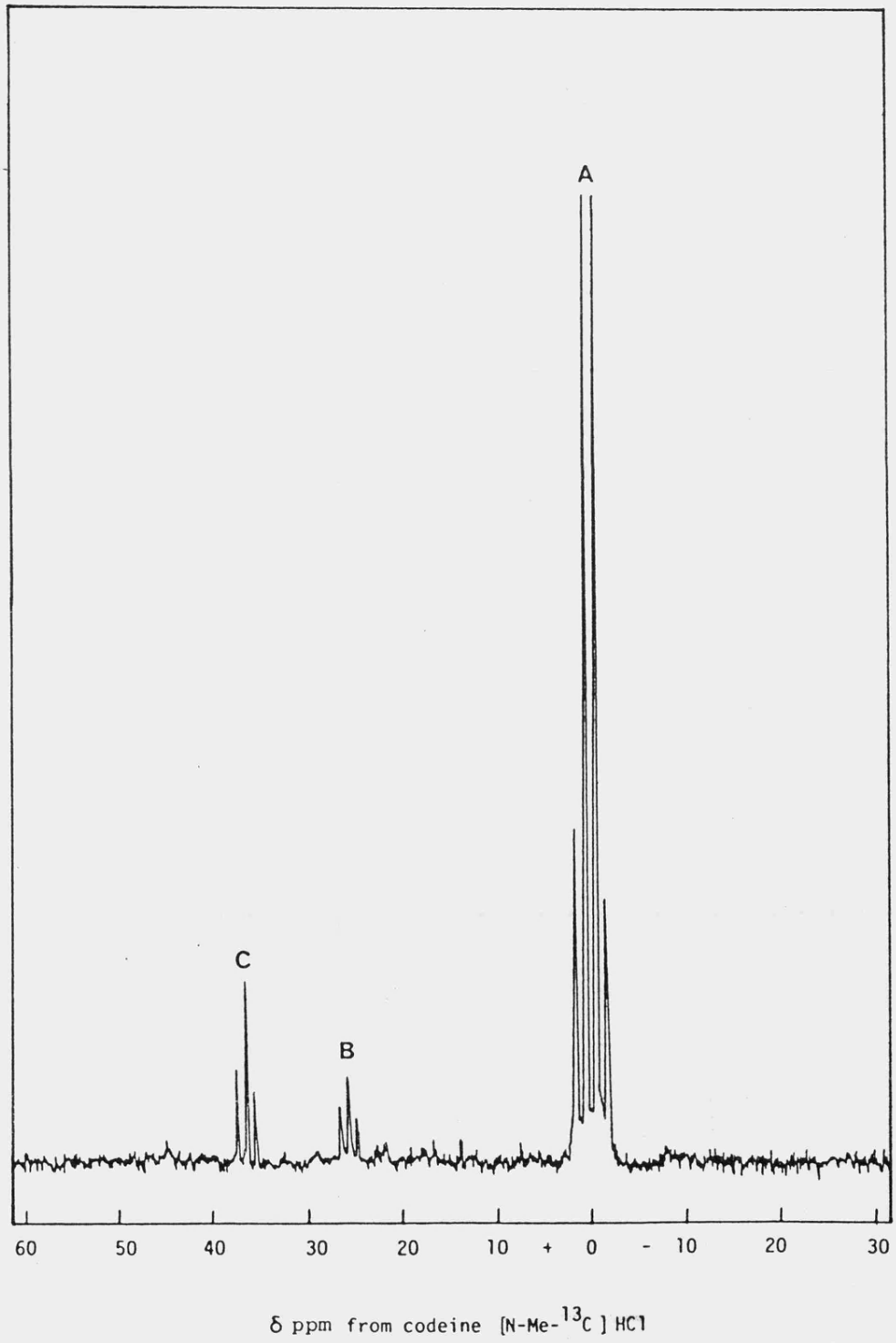


Figure 6.6 shows the OFR spectrum of the same transformation mixture at 23°C, accumulated over alonger period of time (28,200 pulses). The codeine $\left(\text{N-Me-}^{13}\text{C}\right)$HCl signal (A) was observed to be a quartet, typical of the splitting pattern of an N-methyl group. The sulphite adduct (B) was a triplet due to the methylene group present, and the signal at + 26 ppm (C) was also a triplet implicating that it also was a methylene group (CH_2).

6.4.6 ^{13}C NMR spectroscopy of enzyme transformation mixtures at various temperatures

Chemical shifts of the N-methyl carbons of codeine $\left(\text{N-Me-}^{13}\text{C}\right)$HCl and the formaldehyde-sulphite adduct were determined, in the presence of the test mixture components, at temperatures between 5°C and 40°C., employing similar test-mixtures to those described in Section 6.4.5.

The results at the extremes of the temperature range (5°C and 40°C), and the values obtained previously at 23°C (page 247). are shown in Table 6.3. These results demonstrated that the chemical shift values did not vary significantly over the temperature range used.

^{13}C NMR transformation experiments with active cell-free extracts and the test mixture described in Section 6.4.5 were therefore performed., in both the COM and OFR mode of operation, at different temperatures in the range 5 - 40°C. ^{13}C NMR spectra similar to those shown in Figures 6.5 and 6.6 were obtained.

Table 6.3 Data from ^{13}C COM spectra of formaldehyde-sulphite adducts at various temperatures

Temperature of NMR probe (^{o}C)	CHEMICAL SHIFTS ppm TMS REFERENCE		CODEINE REFERENCE
	Codeine (N-Me-^{13}C) HCl	Sulphite adduct	Sulphite adduct
$5^{o}C$	41.5	78.3	+36.8
$23^{o}C$	41.6	78.8	+37.2
$40^{o}C$	41.3	78.3	+37.0

Table 6.4. Chemical shifts of signals relative to codeine (0 ppm) produced in enzyme transformation mixtures at various temperatures for the transformation of codeine by *C. bainieri* at pH 7.0

Temp. of NMR probe (°C)	Chemical shift of species ppm (relative to codeine)	OFR splitting pattern
5	+7.2	?[3]
	+25.95	triplet
	+36.8	triplet
10	+25.99	not recorded
	+36.96	not recorded
23	+12.3	?
	+26.1	triplet
	+37.2	triplet
	+43.4	?
27	+14.8	triplet?
	+26.0	triplet
	+36.96	triplet
30	+26.1	not recorded
	+36.9	not recorded
40	+26.2	triplet
	+37.0	triplet

NB. 1. Codeine N-oxide + 16.7 ppm relative to codeine (quartet)

2. The protein concentration determined for each cell-free extract varied from 0.75 to o.1 mg ml^{-1}.

3. ? indicates that the splitting pattern could not be interpreted

Data from these spectra are shown in Table 6.4, and discussed later on page 258.

6.4.7 ^{13}C NMR spectroscopy of control enzyme transformation mixtures at various temperatures

The NMR experiments were repeated at each of the temperatures in the range 5°C to 40°C, using codeine (N-Me-^{13}C)HCl and the test mixture component described in Section 6.4.5, with cell-free extract which was boiled for 10 minutes prior to its use. ^{13}C NMR spectra of control transformation mixtures containing boiled enzyme showed no product or intermediate peaks at any of the temperatures used.

6.5 Discussion

It was necessary to determine the chemical shifts for carbon atoms of the N-methyl group of codeine, and subsequent intermediate N-substituent groups, which may arise during codeine N-demethylation by *C. bainieri*. The assignments were required for identification purposes in subsequent enzyme transformation experiments. Chemical shift values of 41.5 ppm and 58.3 ppm were obtained for the N-methyl groups of codeine HCl, and codeine N-oxide respectively, with TMS as reference (0 ppm).

For the enzyme-transformation studies it was desirable that the free formaldehyde, produced by the microbial N-demethylation of

codeine, was 'trapped' by a suitable reagent included in the reaction mixtures. The trapping agent - formaldehyde product was required to possess one signal in the COM NMR spectra, well displaced from the codeine $\left(\text{N-Me-}^{13}\text{C}\right)$HCl and possible intermediates in the N-demethylation reaction. Signals from ^{13}C NMR spectra determined for ^{13}C labelled formaldehyde with various trapping agents, selected for their ability to react rapidly with aldehydes[153] were shown in Table 6.1 . The chemical shifts of the N-$^{13}CH_3$ of codeine HCl and codeine-N-oxide were included for comparison.

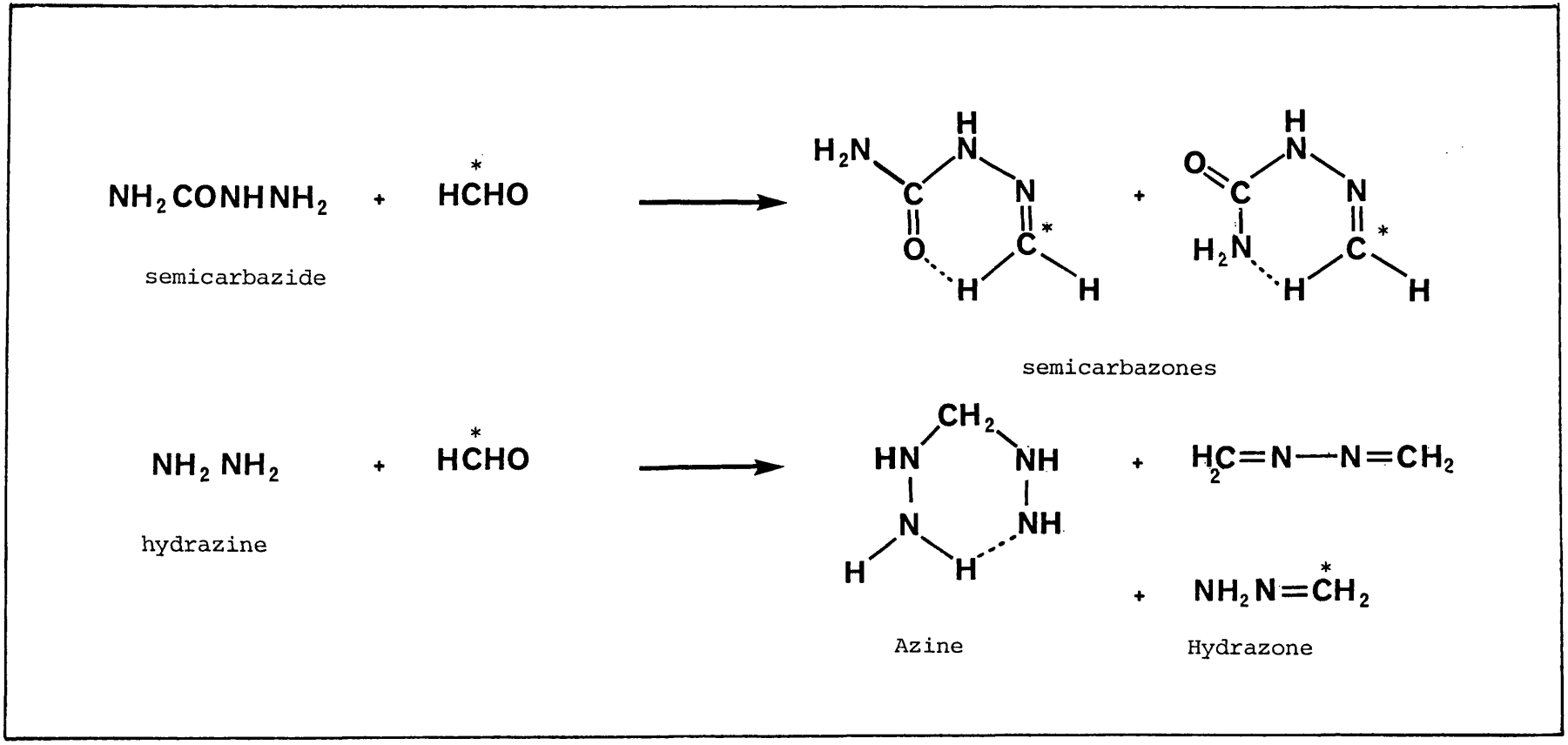
All the trapping agents investigated, except semicarbazide and hydrazine, produced a single resonance, in the COM NMR spectra. Semicarbazide and formaldehyde reacted to form a semicarbazone and produced a doublet at 56.9 ppm and 59.9 ppm. Both signals were triplets in the OFR spectra implicating methylene groups (CH_2). The duplicity of signals was believed to occur because the semicarbazone can form two different cyclic structures which may be stabilised by hydrogen bonds (see Figure 6.7). The magnetic environment of the methylene group in each ring is different thus affording different chemical shifts. In a similar manner the reaction of formaldehyde with hydrazine affords three signals (Table 6.1) arising from the hydrazine, and possibly a further reaction with a second molecule of formaldehyde, or of hydrazine to give an azine[154] illustrated in Figure 6.7.

Alcohol dehydrogenase (A.D.H.) in the presence of the co-factor NADH, converted formaldehyde to methanol:-

Figure 6.7. Possible conformations of the formaldehyde products with semicarbazide and hydrazine

* denotes ^{13}C enriched carbon

--- denotes hydrogen bond



$$\overset{*}{H}CHO + NADH + H^{+} \xrightarrow{ADH} \overset{*}{C}H_3OH + NAD^{+}$$

The ^{13}C enriched methanol produced a singlet at 49.5 ppm in the ^{13}C NMR spectra (Table 6.1)

A furthere requirement was that the trapping agent should not interfere with the N-demethylation process at the concentration used. Determination of the transformation activity of the cell-free extract, in the presence of each trapping agent, produced the results shown in Table 6.2. The transformation activity in the mixture incorporating semicarbazide was assumed to be 100%, since no evidence of enzyme impairment by this compound was found in previous studies[18]. Comparison of the activities of the mixtures containing the other trapping agents revealed that only phenylhydrazine significantly impaired the N-demethylation of codeine. The alcohol dehydrogenase system was undesirable as the methanol signal at 49.5 ppm might conceal intermediates in this region of the NMR spectrum. Since both semicarbazide and hydrazine produced multiplet peaks in the COM NMR spectra, the most suitable trapping agents were sodium sulphite or sodium bisulphite. The sulphite was preferred, since kinetic studies have shown that it reacts with formaldehyde much more rapidly than bisulphite[155], and its signal in the COM NMR spectrum was further downfield from codeine $\left(\text{N-Me-}^{13}\text{C}\right)$HCl, so it was less likely to obscure intermediate signals in the N-demethylation process.

Determination of the chemical shifts for codeine $\left(\text{N-Me-}^{13}\text{C}\right)$HCl

and the formaldehyde-sulphite adduct in the presence of all the test mixture components, at the operating temperature of the instrument (23°C) revealed that these values were not significantly affected by the presence of the other components in the test mixture.

The ^{13}C NMR transformation experiment was therefore performed initially at the operating temperature of the instrument (23°C). Single resonances in the COM NMR spectra of 41.5 ppm and 78.8 ppm (with reference to TMS) were attributed to codeine(N-Me-^{13}C)HCl and the formaldehyde-sulphite adduct respectively. In the OFR NMR spectra the codeine(N-Me-^{13}C)HCl signal ((A) in Figure 6.6) produced a quartet typical of the splitting pattern of an N-methyl group, and the formaldehyde sulphite adduct signal ((B) in Figure 6.6) produced a triplet characteristic of the methylene group present.

The rate of an enzyme catalysed reaction can be markedly influenced by temperature. At sub-optimum temperatures the reduced rate of the reaction may enable transient intermediates to appear for longer periods of time. Sewell[18] demonstrated that codeine N-demethylation by cell-free extracts from *Cunninghamella* species exhibited a broad optimum between 30 and 32°C. Enzyme denaturation occurred at temperatures above 40°C, and the optimum temperature for the growth of the organism was 27°C. Further ^{13}C NMR transformation experiments were therefore performed over a temperature range of 5°C to 40°C. Determination of the chemical shifts for codeine(N-Me-^{13}C)HCl

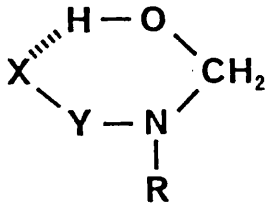
and the formaldehyde-sulphite adduct, in the presence of all the test mixture components, revealed that these values did not vary significantly over the temperature range used (Table 6.3). The results of subsequent enzyme transformation experiments (Table 6.4) demonstrated that codeine was N-demethylated at all the temperatures studied. The signals which were triplets at about + 37 ppm (relative to codeine), confirmed the presence of the formaldehyde-sulphite adduct. Various possible intermediate signals were also observed, but only one occurred in all the spectra at each temperature (Table 6.4). This was a triplet in the OFR spectra, in each case centred at +26 ppm relative to codeine.

^{13}C NMR spectra of control transformation mixtures containing boiled cell-free extract, showed a resonance at about 41.5 ppm corresponding to codeine $\left(\text{N-Me-}^{13}\text{C}\right)$HCl, but no product or intermediate peaks at any of the temperatures used. This confirmed that the signals observed in the active transformation mixtures were caused by N-demethylase activity and not by chemical breakdown of codeine in the NMR tube. The two proposed mechanisms for the N-dealkylation of tertiary amines were described in Chapter 5 of this thesis, and illustrated in Figure 5.1. In addition, it was concluded in Chapter 5 that the tertiary amine, codeine, was N-demethylated probably by a cytochrome P450 type mixed function oxidase extracted from *Cunninghamella bainieri*, in the presence of molecular oxygen, to yield the secondary amine, norcodeine and formaldehyde.

If N-demethylation of codeine was occurring by N-oxidation an N-methyl signal in the OFR spectra would be seen as a quartet centred at 58.3 ppm (or +16.7 ppm relative to codeine) and no such signal was observed (Table 6.4). Alternatively, the mechanistic route might involve a carbinolamine intermediate[133] generated by direct α-carbon oxidation, with an N-hydroxymethyl function giving a triplet in the OFR spectrum. The triplet at +26 ppm (relative to codeine) in Table 6.4 may well correspond to this. However, confirmation of this cannot be obtained by chemical synthesis since N-(hydroxymethyl)-codeine is unstable.

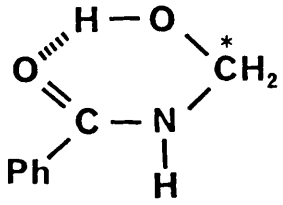
Comparison of the data with those for other compounds containing the carbinolamine moeity ($N-CH_2-OH$) was not possible since stable carbinolamines are rare, and for those that are known there is no ^{13}C NMR data available. Gorrod and Temple[156] have reported a series of carbinolamines found as stable metabolites in various mammalian metabolic studies. A prerequisite for their stability appears to be the presence of an electron withdrawing group (such as carbonyl) adjacent to the nitrogen (Figure 6.8). The stability is afforded by the formation of hydrogen bonds between the electron withdrawing group X, and the hydroxyl group to form a ring structure[156].

In the search for possible anti-tumour agents, Stevens[157], has synthesised a series of stable N-(hydroxymethyl) compounds related to formamide. Values for the N-(hydroxymethyl) carbon for two of these compounds are listed in Figure 6.8.



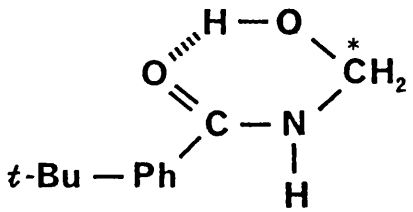
Basic structure of a stable carbinolamine

Y—X = electron with-drawing group



N-(hydroxymethyl)-benzamide

δ_C ($\overset{*}{C}H_2$) 63.0ppm TMS ref.,+21.5ppm Codeine ref.



4-(t-butyl)-N-(hydroxymethyl)benzamide

δ_C ($\overset{*}{C}H_2$) 69.66ppm TMS ref.,+28.16ppm Codeine ref.

Figure 6.8. Chemical shifts of stable N-(hydroxymethyl) compounds.
(* denotes ^{13}C enriched carbon)

The nitrogen of the codeine carbinolamine intermediate is tertiary and part of the phenanthrene ring, with no electron withdrawing group adjacent to the nitrogen to stabilise it, so the chemical shift of the carbon is likely to be slightly different to the values shown in Figure 6.8. However, all these values are similar and suggest, but cannot confirm, that the intermediate at +26 ppm relative to codeine (67 ppm ref. to TMS) observed during the microbial N-dealkylation of codeine, could be the carbinolamine proposed. This is consistent with the results of enzyme kinetic, induction and inhibitor experiments performed in the previous chapter (5).

CHAPTER SEVEN

A STUDY OF THE N-DEALKYLATION OF A SERIES OF 1.4-BENZODIAZEPINE COMPOUNDS BY C. bainieri AND SOME OF THEIR STRUCTURAL AND PHYSICAL PROPERTIES.

CHAPTER 7

7.1 Introduction

Studies with both mammalian[26,133] and microbial preparations[24,25,28] have shown that a large variety of compounds of very different chemical structure can be N-dealkylated by monooxygenases. However, although substrate structure transformation activity studies have been extensively reported for hepatic systems[159-163], very few have been reported for microbial systems. It is generally accepted that the relative rate of oxidative metabolism of a substrate by liver microscomal monooxygenases is related to the substrate lipid solubility[159,161]. This is because the enzyme system is thought to be surrounded by a lipoidal barrier which is penetrable only by compounds with high oil-water partition coefficients. This concept has resulted in several studies relating partition coefficients to rates of metabolism with varying degrees of success[160-162]. For example, McMahon[159] found a rough correlation between the partition coefficients of 6 N-methylamines and their rate of N-dealkylation by rat liver microsomes. Further studies by Hansch *et al.*[164] showed that the relative rates of N-demethylation of these tertiary amines was indeed related to the partition coefficient, and the data could be expressed by equation 7.1;

$$\log BR = 0.470 \log P - 0.268 (pKa - 9.5) - 1.305 \qquad 7.1$$

$$n = 18,\ r = 0.89\ \ s = 0.222$$

where n is the number of points used in the regression analysis, r is the correlation coefficient, s is the standard deviation, BR is

the biological response (i.e. the relative rate of *in vitro* N-demethylation by rat liver microsomes), P is the octanol-water partition coefficient, and Ka is the ionization constant. It was concluded by Hansch *et al.*[164] that both the partition coefficient and the pKa were statistically significant predictors of the relative rate of N-demethylation. The strength of the base is thought to regulate the amount of substrate available for distribution between the buffered aqueous phase and the lipid phase. Its partition is a function of its state of ionization, so the neutral form of the base would be expected to have a substantially higher partition coefficient than the ionized form. In support of this view, McMahon and Easton[160] showed that the rate of N-demethylation of a series of aliphatic amines increased with decreasing base strength, and concluded that these results were influenced by the effect of base strength on lipid solubility[160].

Another variable which may affect the rate of N-dealkylation of a substrate is the steric hindrance caused by an increase in branching on the carbon atoms attached to the nitrogen. For example, La Du *et al.*[165] observed a decrease in the rate of N-dealkylation of a group of aminopyrine homologues when the alkyl chain length was increased from methyl to n-butyl, despite the increases in the lipophilicity of the substrate. Contrary to this, Zeigler *et al.*[166] demonstrated that tertiary amines containing N,N-dimethylalkylamine moieties with 8-12 carbons in the side chain were oxidised faster than compounds with shorter alkyl chains.

The ability of a compound to partition between a relatively non-polar solvent and water is normally used as a measure of its hydrophobic-hydrophilic character, and expressed as the partition coefficient (P). This parameter is commonly determined by means of an octanol and water partition system. However, the work of Martine and Synge[167], and Consden *et al.*[168] in establishing relationships between the Rf values obtained from partition chromatography and the partition coefficient, has provided an alternative to octanol-water partition. Martin[169] deduced on theoretical grounds that for partition chromatography on paper or lipid impregnated plates:-

$$\log P = aRm + b(\log C) \qquad 7.2$$

where P is the partition coefficient, C is a constant for the system, and Rm is the log of (1/Rf) - 1, where Rf is the retention of the system. The use of this linear relationship to predict partition coefficients from chromatographic data has been extensively reviewed by Tomlinson[170]. Furtheremore, as the column capacity ratio (κ) used to describe solute retention in HPLC (see Equation 1.2) is analogous to the Rm value, the partition of a compound between a HPLC stationary phase and mobile phase can be given by:

$$\log P = a \log K + b \qquad 7.3$$

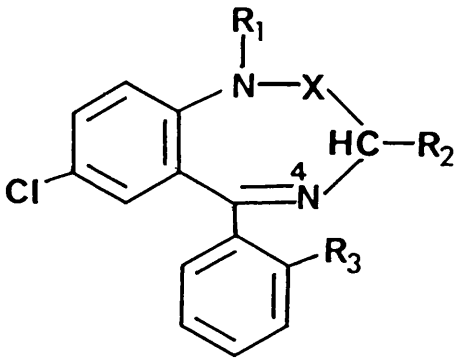
McCall[171] chromatographed a wide variety of chemical compounds with known log P values(determined from octanol-water studies) on a Corasil C-18 reversed phase HPLC column. He confirmed the relationship between P and κ in Equation 7.3, providing the process was predominantly one of partition. A vigorously silylated

column generally gave better correlations than an untreated column except in the case of the more bulky compounds examined. These were probably sterically hindered from reaching the available silanol sites. Some of the potential advantages of using chromatography to replace the often tedious octanol-water partition coefficient measurements have been described[171]. These include the speed and reproducibility of the HPLC technique, and because solvent lipophilicity can be rapidly adjusted, compounds whose partition coefficients vary substantially can be quickly measured. Samples need not be pure since contaminants do not interfere with κ determinations and most compounds can be determined as long as they can be chromatographed and detected[171].

Diazepam, a member of the 1,4-benzodiazepine group of minor tranquiliser drugs, is only very slightly soluble in water[172]. However, several species of *Cunninghamella* were reported by Sewell[18] to N-demethylate this compound with comparatively high transformation yields. Therefore, it was envisaged that other 1,4-benzodiazepine compounds structurally related to diazepam may also be N-dealkylated by *Cunninghamella* sp. It was envisaged that the extent of transformation could be related to the relative lipid solubility and base strength of the substrate.

In the present study, a selection of 1,4-benzodiazepine compounds, structurally related to diazepam, were examined for their transformation yield when incubated with growing cell cultures of *Cunninghamella bainieri* over a given time period. Reversed phase HPLC was employed to determine the column capacity ratios (κ) for

Table 7.1. Structural variations of 1,4-benzodiazepines selected for microbial transformation studies; chlordiazepoxide (1), temazepam (2), diazepam (3), flurazepam (4) and medazepam (5)



	R_1	R_2	R_3	X
1.*	-	H	H	=C-NH(CH_3)
2.	CH_3	OH	H	C=O
3.	CH_3	H	H	C=O
4.	CH_2CH_2-N(Et)$_2$	H	F	C=O
5.	CH_3	H	H	CH_2

* Chlordiazepoxide (1) contains an N-oxide group (N→o) at position 4.

each compound. Since the κ value has been shown to be directly related to the partition coefficient (P)[171] (Equation 7.3) it should be possible to observe whether the amount of substrate transformed is related to the κ value, and hence the lipid solubility.

The benzodiazepine compounds selected for this study (shown in Table 7.1) are members of an important group of psychotherapeutic drugs which possess sedative-hypnotic and tranquillizing properties[173]. They all possess a characteristic seven membered diazepine ring with nitrogens in position 1 and 4. Chlordiazepoxide (1 in Table 7.1) contains an N-oxide group at position 4, and a methylamino group at position 2. Unlike the other compounds studied the N-methyl group of chlordiaxepoxide is presented as a secondary amine. The N-methyl functions of temazepam (2) and diazepam (3) are presented as cyclic amides, but temazepam differs from diazepam in the presence of a hydroxyl group at position 3. Flurazepan (4) was included in the series to investigate the capacity of *C. bainieri* to remove more complex N-alkyl functions, and medazepam (5) contains a tertiary amine group (position 1) with no additional functional groups on the diazepine ring.

7.2 Materials

7.2.1 Transformation substrates

Chlordiazepoxide hydrochloride, Diazepam, Flurazepam, dihydrochloride and medazepam; were gifts from Roche Products Ltd.,

Welwyn Garden City.

Temazepam; Extracted from Normison capsules (Wyeth Laboratories, Maidenhead, Berks). The contents of 20 capsules (20 mg) were added to 2 N HCl (5.0 ml), neutralised to pH 7.0 with 2 N NaOH, and extracted into diethylether (4 x 10 ml). The organic phase was separated, dried (anhydrous $MgSO_4$) and evaporated to dryness to yield temazepam free base (360 mg).

7.2.2 Reference standards

Nor- methylchlordiazepoxide, normethyldiazepam and desalkyl flurazepam; Roche Products Ltd.

Normethyl-temazepam (Oxazepam); Extracted from serenid tablets (Wyeth Laboratories). 30 tablets (15 mg) were crushed and dissolved in 2 N HCl (25 ml). The solution was filtered, basified (2 N NaOH) to pH 8.0, and extracted into chloroform (4 x 25 ml). The organic phase was separated, dried (anhydrous $MgSO_4$) and evaporated to dryness to yield the nor-temazepam free base (410 mg).

Nor-methylmedazepam; This was prepared by adapting the method used by Montzka *et al.*[10] A solution of 2,2,2-trichloroethylchloroformate (0.4 ml) in dry toluene (2 ml) was added dropwise to a mixture of medazepam (0.5 g), very dry Na_2CO_3 (0.5 g) and dry toluene (6 ml) contained in a 50 ml round bottomed flask. The mixture was stirred vigorously at room temperature for 24 hours. Diethyl-ether (10 ml) was added, and the whole was washed with 2 N HCl (2 x 10 ml) and water (10 ml). The organic layer was separated, dried (anhydrous $MgSO_4$) and evaporated to leave an oil, which was dissolved

in glacial acetic acid (20 ml) and refluxed with zinc dust (2 g) for 1 hour. The mixture was then stirred at room temperature for a further 4 hours, poured onto ice, basified (5 N NaOH), and extracted into chloroform. The organic layer was dried (anhydrous $MgSO_4$) and evaporated to give pale yellow solid (220 mg).

Analytical; ^{13}C NMR ($CDCl_3$; normedazepam); 50.65 (OFR t; C_3); 63.85 (OFR t; C_2); 118.10 (OFR d; C_9); 125.8 (OFR s; C_7); 128.4 (OFR m; $C_{3'}$, $C_{5'}$), 128.9 (OFR m; $C_{2'}$, $C_{6'}$); 129.33 (OFR s; C_{5A}); 129.60 (OFR d; C_6); 130.06 (OFR d; C_8); 130.75 (OFR d; $C_{4'}$); 139.73 (OFR s; $C_{1'}$); 148.85 (OFR s; C_{9A}); 171.78 (OFR s; C_5). These data are consistent with the structure of nor-medazepam.

7.2.3 HPLC stationary phase and internal standards; LiChrosorb-RP-2, 5 µm particle size, was purchased from Jones Chromatography Glamorgan.

Nitrazepam; British Pharmacopoeia Commission, London,

Phenacetin (Analar) - BDH Ltd.

7.3 Methods

7.3.1 HPLC assay methods.

Quantitative HPLC assay procedures were required for each transformation product and its corresponding nor-metabolite. Some of the benzodiazepine compounds were so strongly retained on existing HPLC columns (Partisil-10 ODS-2 in Section 2.4.4, ODS-Hypersil 5 µm

in Section 2.4.5) resulting in poor chromatography. To reduce the retention times a smaller column was employed (5 cm x 4.6 mm i.d.), packed with LiChrosorb RP-2 reversed phase material, a stationary phase of silica bonded with a hydrocarbon chain 2 carbons long, as opposed to 18 in the ODS materials listed above.

(a) Column packing and testing; A stainless steel column (5 cm x 4.6 mm i.d.) was packed with LiChrosorb RP-2, 5 μm particle size, by the method described in Section 2.3.2 (page 54). The packed column was subjected to the column tests described in Section 2.3.2, page 54 to evaluate the quality of the packing and the column efficiency, as described by h. (Equation 1.5), was found to be 5.5.

(b) HPLC conditions;10 μl volumes of samples were injected onto the LiChrosorb RP-2 HPLC column. Each assay was performed at 40°C, maintained by immersing the mobile phase reservoir, column and injector into a thermostatically controlled water bath. The mobile phase flow rate through the column was 2 ml min^{-1}, and the remaining operating conditions for each assay are shown in Table 7.2.

Typical HPLC chromatograms demonstraing the resolution of substrate, internal standard and transformation product in each assay are shown in Figures 7.1 and 7.2.

(c) Construction of calibration plots; Stock solutions of each transformation substrate/transformation product were prepared

Table 7.2. HPLC conditions for the assay of substrate and transformation products in microbial transformation mixtures

	A. Chlordiazepoxide/ Norchlordiazepoxide	B. Temazepam/ Nortemazepam	C. Diazepam/ Nordiazepam	D. Flurazepam/ Desalkylflu-razepam	E. Medazepam/ Normedazepam
Internal standard	Phenacetin	Phenacetin	Nitrazepam	Diazepam	Nordiazepam
Wavelength of UV detector (nm)	240	240	240	230	240
Mobile phase:					
Acetonitrile %;	27	28	30	35	35
Phosphate buffer (mM);	4.4	4.4	6.6	6.6	6.6
pH;	6.4	6.4	6.0	6.4	6.2
Pentane sulphonic acid (mM), pairing ion	5.0	5.0	-	5.0	-

Figure 7.1. HPLC chromatograms obtained in the assay of A) Chlordiazepoxide, B) Temazepam and C) Diazepam and their respective N-demethylated transformation products for the conditions shown in Table 7.2.

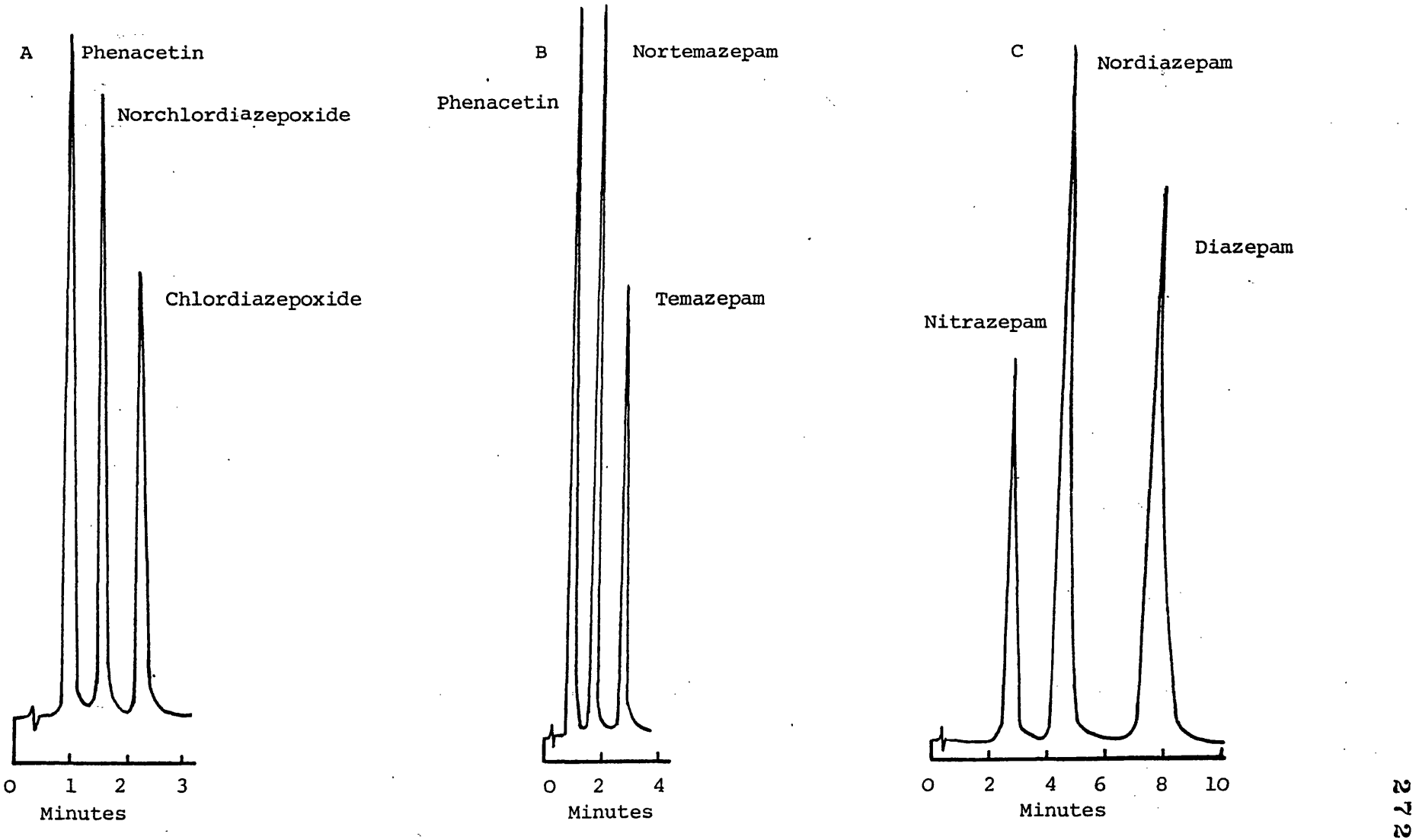
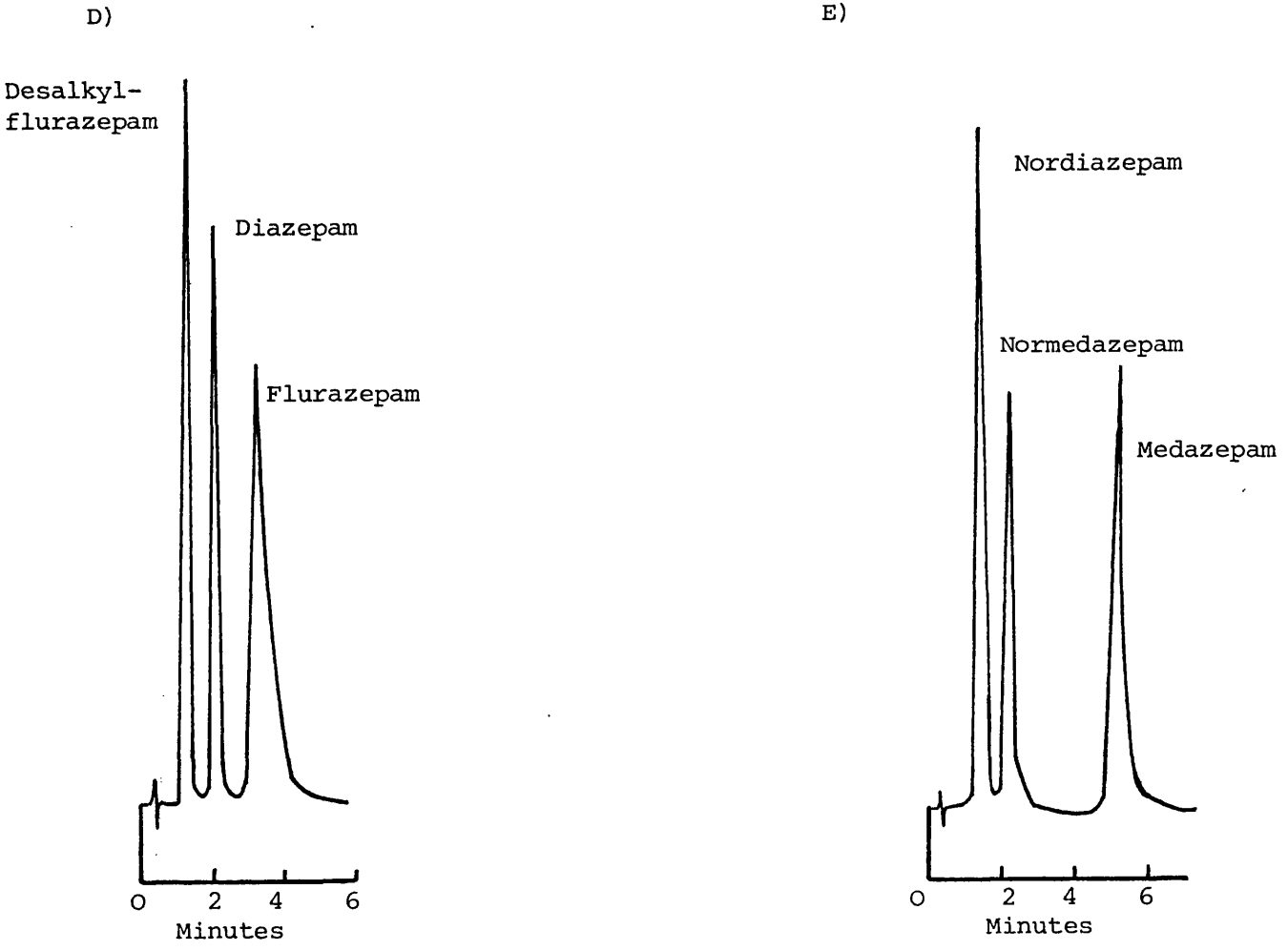


Figure 7.2. HPLC chromatograms obtained in the assay of D) Flurazepam and E) Medazepam and their respective N-demethylated transformation products for the conditions shown in Table 7.2.



in the appropriate mobile phase (Table 7.2). Stock solutions containing the respective internal standard in mobile phase were also prepared for each assay. Stock solutions were then serially diluted with the same mobile phases to produce a series of calibration solutions for each assay in the concentration ranges shown in Table 7.3, each incorporating the internal standard at the particular concentration shown in Table 7.3. Samples of each calibration solution (10 µl) were assayed in triplicate by the HPLC procedure described above (see Table 7.2).

Calibration plots for each system (A to E) were constructed from the ratio of the peak heights of the analyte to the internal standard obtained at each analyte concentration. Each plot was subjected to regression analysis to provide the data shown in Table 7.3. These data were used to calculate the transformation and specific transformation from HPLC peak ratios obtained in the analysis of transformation mixture extracts.

(d) Precision of the detector response; This was determined for each HPLC assay system by chromatographing a calibration solution of test substrate and transformation product(from the middle of the calibration range) ten times under the conditions described in Table 7.2. The results obtained for the peak heights of the analytes from 10 injections are shown in Table 7.4, and demonstrate that duplicate injections provide adequate precision.

Table 7.3. Data obtained from the linear regression analysis of HPLC calibration plots for the peak height ratio (benzodiazepine to internal standard) against benzodiazepine concentration in the respective calibration solution.

HPLC system	Concentration range (mg ml^{-1})	Slope	Intercept	Correlation coefficient
A. Chlordiazepoxide	0.003 to 0.018	100.6	-0.0152	0.9998
Norchlordiazepoxide	0.002 to 0.012	126.3	-0.0048	0.9999
Phenacetin*	0.05			
B. Temazepam	0.05 to 0.15	67.9	0.0034	0.9998
Nortemazepam	0.05 to 0.15	124.4	0.0216	0.9999
Phenacetin*	0.1			
C. Diazepam	0.001 to 0.01	140.6	-0.0073	0.9994
Nordiazepam	0.001 to 0.01	226.6	-0.016	0.9994
Nitrazepam*	0.005			
D. Flurazepam	0.08 to 0.4	2.46	-0.0051	0.9997
Desalkylflurazepam	0.025 to 0.125	16.05	-0.0078	0.9999
Diazepam*	0.1			
E. Medazepam	0.01 to 0.1	40.6	0.0036	0.9998
Normedazepam	0.01 to 0.1	55.7	0.0058	0.9998
Nordiazepam*	0.005			

* Internal standard

Table 7.4. Data obtained for the peakheights of 10 injections of the analytes showing the precision of the detector response for each HPLC assay

Analyte	Mean peak height (mm)	Standard deviation	% coefficient of variation (±)	% range of error (±) for duplicate injections
Chlordiazepoxide/	105.9	1.554	1.46	2.07
Norchlordiazepoxide	90.0	1.066	1.18	1.67
Temezaepam/	26.0	1.137	0.52	0.74
Nortemazepam	36.2	1.157	0.43	0.61
Diazepam/	55.2	1.378	2.49	3.53
Nordiazepam	88.5	0.32 1	0.36	0.51
Furazepam/	34.7	0.129	0.37	0.52
Desalkylflurazepam	68.5	0.233	0.34	0.48
Medazepam/	86.0	1.414	1.64	2.33
Normedazepam	148.7	1.202	0.81	1.15

7.3.2 Extraction of substrate and transformation products from transformation mixtures

The aqueous transformation mixtures usually contained a concentration of benzodiazepine test substrate (and its transformation product) of 1 mM in a volume of 50 or 60 ml. These systems were basified and extracted into organic solvent (3 x 50 ml). The organic solvent was separated, dried (anhydrous $MgSO_4$) and evaporated to dryness. The residue was dissolved in the appropriate mobile phase incorporating the internal standard and serially diluted with mobile phase to provide a sample concentration within the HPLC assay calibration range (see Table 7.3). The conditions for the extraction of each substrate and corresponding transformation product and the respective extraction efficiencies, determined by the procedure described in Section 2.7.3 for codeine and norcodeine, are shown in Table 7.5.

Table 7.5. Conditions for the extraction of benzodiazepine test substrates and their transformation products from transformation mixtures, and the extraction efficiencies obtained under these conditions.

Test substrate/ transformation product	Base	Extraction solvent	Extraction efficiency(%)
Chlordiazepoxide/ Norchlordiazepoxide	-	Not extracted	-
Temazepam/ Nortemazepam	Neutralised to pH 7.0 with 2 N NaOH	Diethyl-ether	93.2 95.6
Diazepam/ Nordiazepam	2N NaOH	Chloroform	92.5 96.5
Flurazepam/ Desalkylflurazepam	2N NaOH	Chloroform	95.1 98.6
Medazepam/ Normedazepam	2N NaOH	Chloroform	95.4 97.9

7.4 Experimental

7.4.1 Microbial transformation of 1,4-benzodiazepine compounds in submerged cultures of *C. bainieri*

C. bainieri was cultured by the two-stage transformation protocol described in Section 3.3.1 (page 133). The stage-two culture flasks containing stage-two growth medium (50 ml) with glucose carbon source (1% w/v), were inoculated with stage-one growth (10 ml) and incubated at 27°C, 250 rpm, for 24 hours. Each benzodiazepine test substrate was then added to stage-two cultures as a filter sterilised solution, to produce a concentration of 1 mM in the transformation mixture. Cultures were incubated under the same conditions for a further 14 days. Control flasks were also prepared containing each test substrate in stage-two growth medium with glucose (1% w/v) and incubated as described above. Sufficient stage-two cultures (including controls) were prepared to enable samples to be taken at 2-3 day intervals during the course of the incubation for the determination of growth (dry cell weight, page 134) and substrate transformation (HPLC). Substrate transformation was expressed as the concentration of transformation product (μM), and specific transformation as the transformation (μ M) per mg dry cell weight of the biomass.

Progress curves constructed from the transformation results for the transformation obtained with temazepam, diazepam and medazepam are shown in Figure 7.3, and for chlordiazepoxide and flurazepam in Figure 7.4(a) and (b) respectively. The final dry cell

Table 7.6. Microbial growth, transformation and specific transformation by *C. bainieri* when incubated with benzodiazepine compounds in stage-two growth medium containing glucose (1% w/v) at 27°C, 250 rpm.

Substrate	Optimal dry cell weight mg ml^{-1}	Transformation (μM)	Specific transformation (μM per mg dry cell wt)	Comments on transformation product(s)
1. Chlordiazepoxide	4.80	772.9 (A)	2.45	Product (A) not normethylchlordiazepoxide
2. Temazepam	2.06	31.4	0.25	Nortemazepam
3. Diazepam	1.99	130.1	1.58	Nordiazepam
4. Flurazepam	3.91	84.6 112.7 (B) 89.5 (C)	-	Desalkylflurazepam and products (B) and (C)
5. Medazepam	3.72	261.5	1.31	Normedazepam

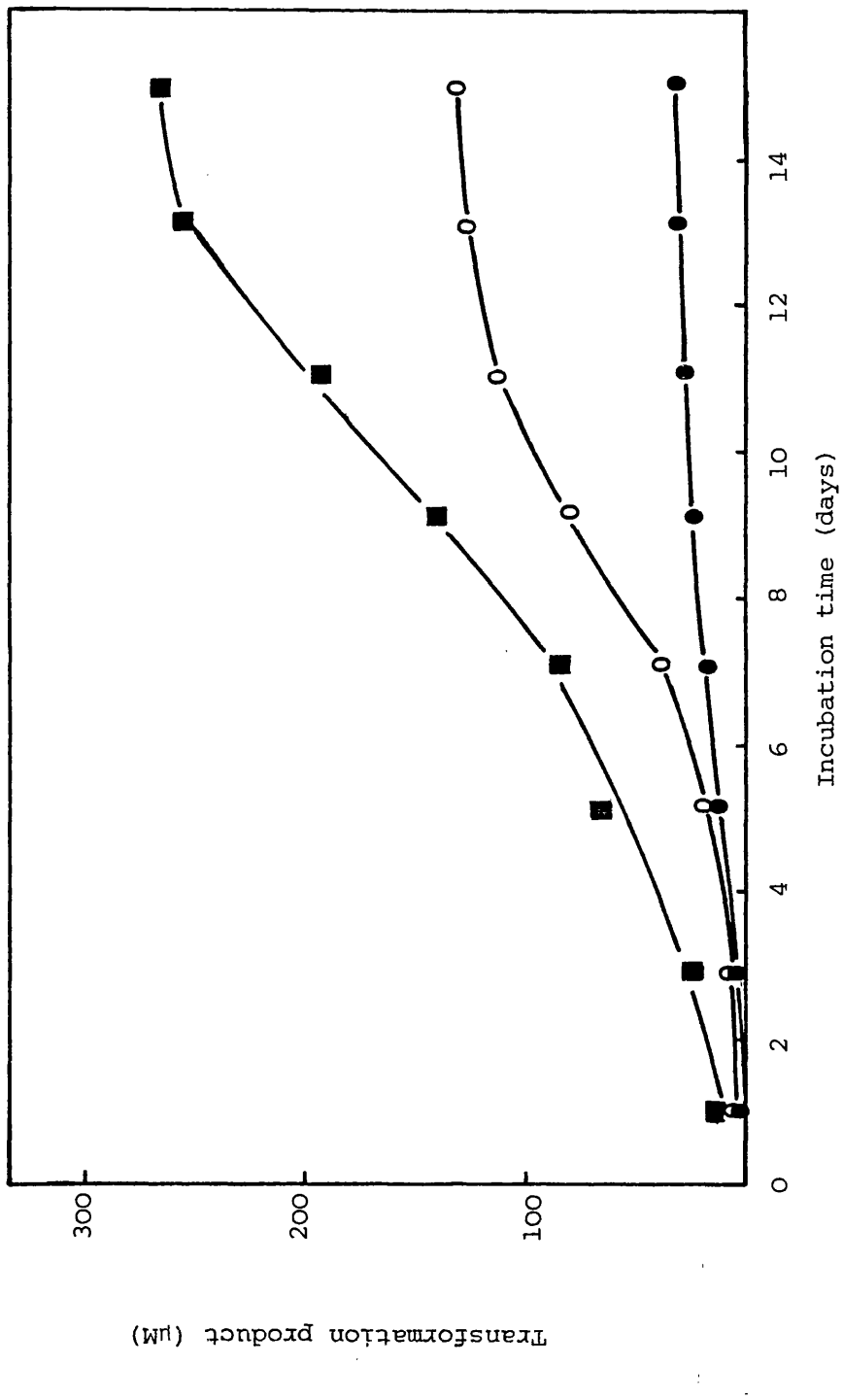
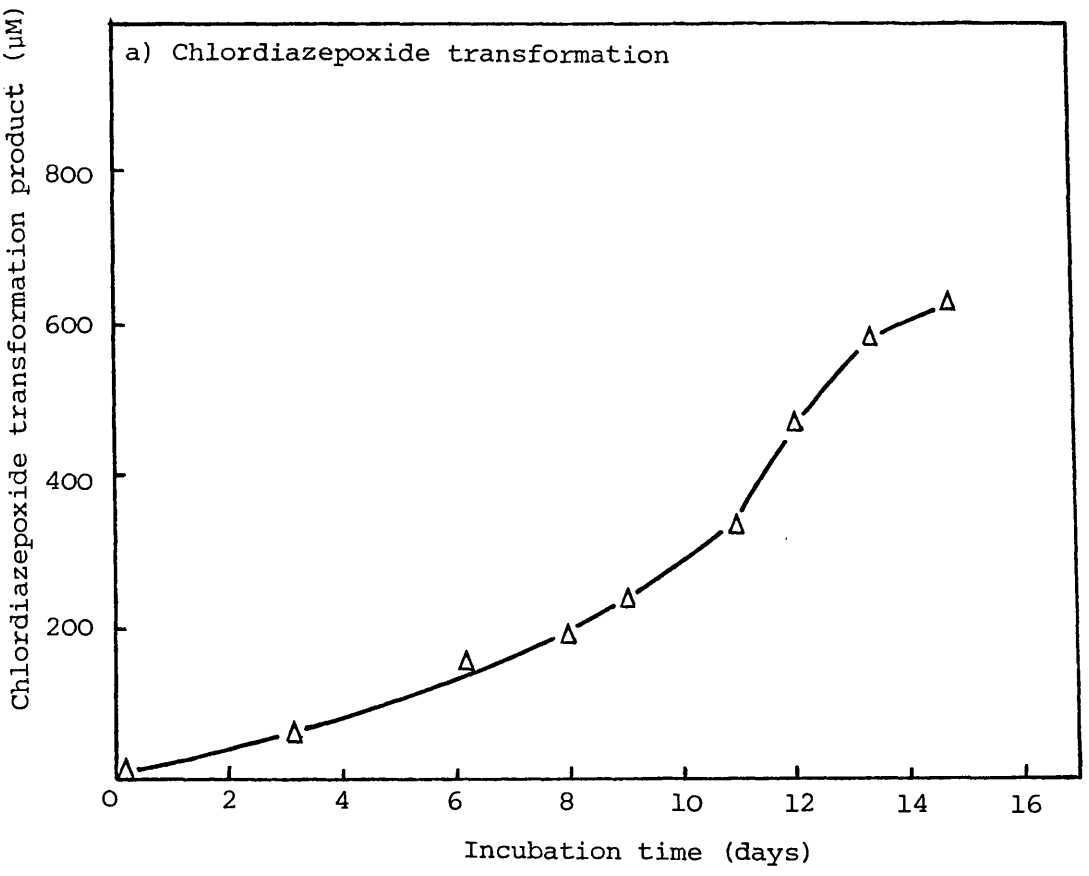
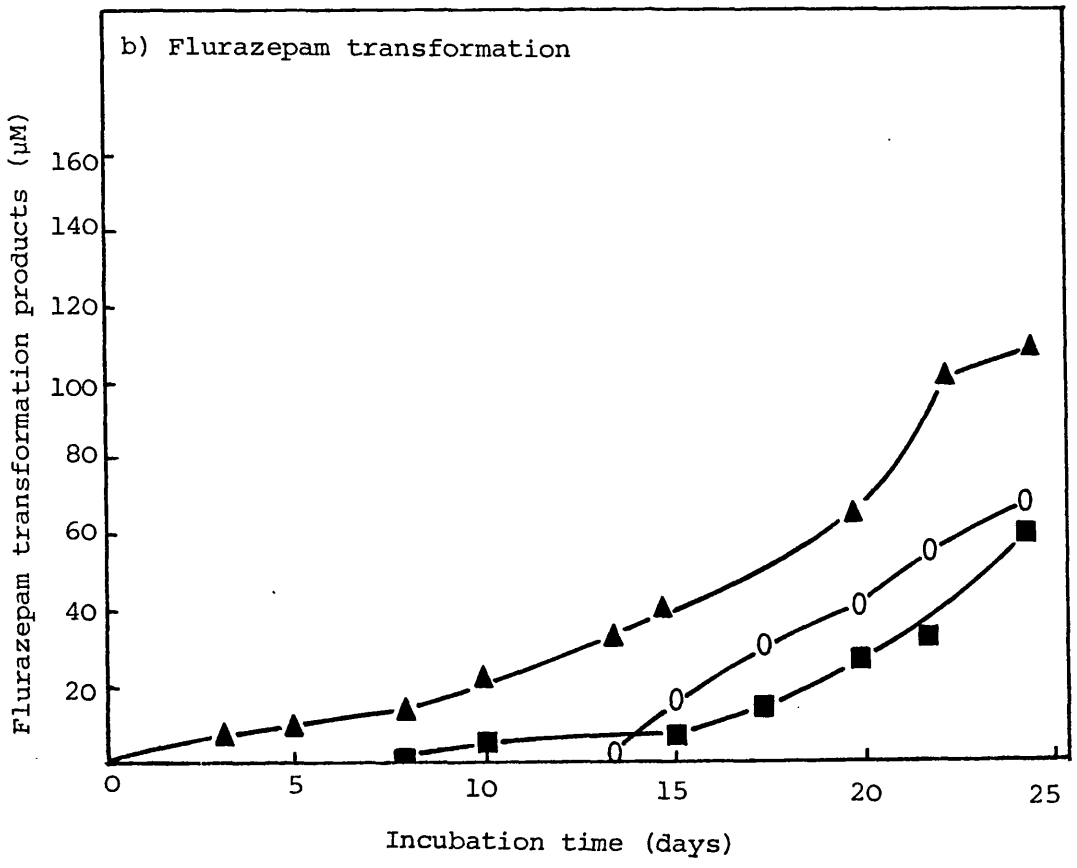


Figure 7.3. Progress curves for the transformation of temazepam ● , diazepam O and medazepam ■ by *C. bainieri* incubated at 27°C , 250 rpm in stage-two growth medium with glucose (1% w/v)

Figure 7.4. Progress curves for chlordiazepoxide transformation (a) and flurazepam transformation (b) by *C. bainieri* incubated at 27°C, 250 rpm in stage-two growth medium with glucose (1% w/v); Chlordiazepoxide transformation product (A) (Δ), desalkyl flurazepam (■), product B (▲), product C (0).


a) Chlordiazepoxide transformation
Chlordiazepoxide transformation product (μM)
800
600
400
200
0
2
4
6
8
10
12
14
16
Incubation time (days)


b) Flurazepam transformation
Flurazepam transformation products (μM)
160
140
120
100
80
60
40
20
0
5
10
15
20
25
Incubation time (days)

weight of the biomass, substrate transformation and specific transformation for each test substrate are shown in Table 7.6.

The results in Table 7.6 show that the test substrates temazepam, diazepam and medazepam each formed their respective normethyl transformation products only. Chlordiazepoxide substrate also formed a single transformation product in high yield. However, this product (A) was found to possess a HPLC retention near to, but not identical with, the retention of normethylchlordiazepoxide standard. For the purposes of this study the concentration of product A in the samples was still estimated from the normethylchlordiazepoxide standard to give an approximation of the transformation yield. The significance of the chlordiazepoxide transformation results are discussed on page 289 . Finally, flurazepam, with its long N-alkyl group, produced three transformation products. Unfortunately, standards for all the potential transformation products of flurazepam were not available, but suggestions as to their possible identity are discussed on page 295.

7.4.2 Determination of the column capacity ratio (κ) of the 1,4-benzodiazepine compounds by reversed phase HPLC

Each of the test substrates was dissolved in propan-2-ol to give sample concentrations of 0.01 mg ml^{-1}. Sample volumes (10 µl) of each benzodiazepine solution were injected onto the LiChrosorb RP-2 HPLC column. Retention times of the benzodiazepines and of the unretained propan-2-ol were obtained from the resulting

chromatograms, and these used to calculate the respective column capacity ratios (κ). (see Equation 1.2, page 26). In the first series of experiments the mobile phase employed consisted of propan-2-ol; 0.066 M phosphate buffer, pH 7.0 (25; 75) and the mobile phase flow rate was 2 ml. min^{-1}. The temperature of the HPLC system was varied from 35°C to 60°C, by means of a thermostatically controlled heated water bath. Retention data obtained were used to construct plots of log column capacity ratio (log κ) against reciprocal absolute temperature (1/T) for each benzodiazepine in the series, shown in Figure 7.5.

In the second series of experiments the temperature was maintained at 40°C. Mobile phases were prepared containing increasing proportions of propan-2-ol (from 20% to 35% v/v) in 0.066 M phosphate buffer,pH 7.0. The retention data obtained for each benzodiazepine sample injected onto the column, with each mobile phase, were used to construct plots of log column capacity ratio (log κ) against the percentage of propan-2-ol in the mobile phase, shown in Figure 7.6. Each plot shown in both Figures 7.5 and 7.6 was subjected to regression analysis to give the constants listed in Table 7.7.

The data in Table 7.7 shows that the 1,4-benzodiazepines are retained on the reversed phase column in the same order each time, for all the experimental conditions investigated. Therefore the plots in Figures 7.5 and 7.6 were extrapolated to predict the κ values of each benzodiazepine at 27°C (the transformation temperature)

Figure 7.5. Plots of log column capacity ratio (log κ) against reciprocal temperature (1/T) for a series of 1,4-benzodiazepines obtained using a mobile phase of propan-2-ol (25); 0.066 M phosphate buffer, pH 7.0, at a flow rate of 2 ml. min^{-1} through a LiChrosorb RP-2 HPLC column. (Chlordiazepoxide (Δ); Temazepam (●); Diazepam (0); Flurazepam (▲) and Medazepam (■)).

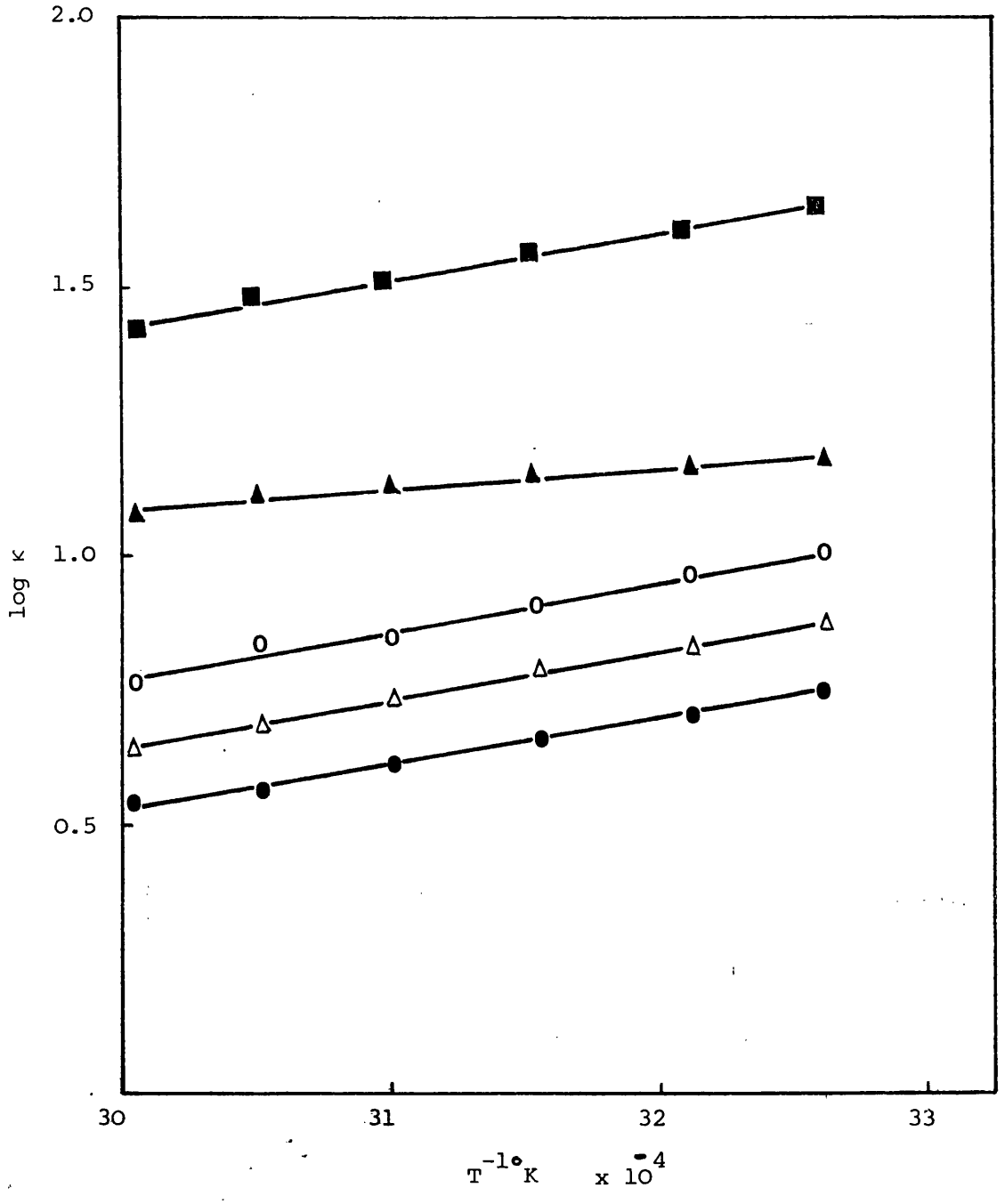


Figure 7.6. Plot of log column capacity ratio (log κ) against the percentage propan-2-ol in the mobile phase for a series of 1,4-benzodiazepines obtained using a LiChrosorb RP-2 HPLC column at 40°C, pH 7.0 and mobile phase flow rate 2 ml. min^{-1}. (chlordiazepoxide (△); temazepam (●); diazepam (○); flurazepam (▲) and medazepam (■)).

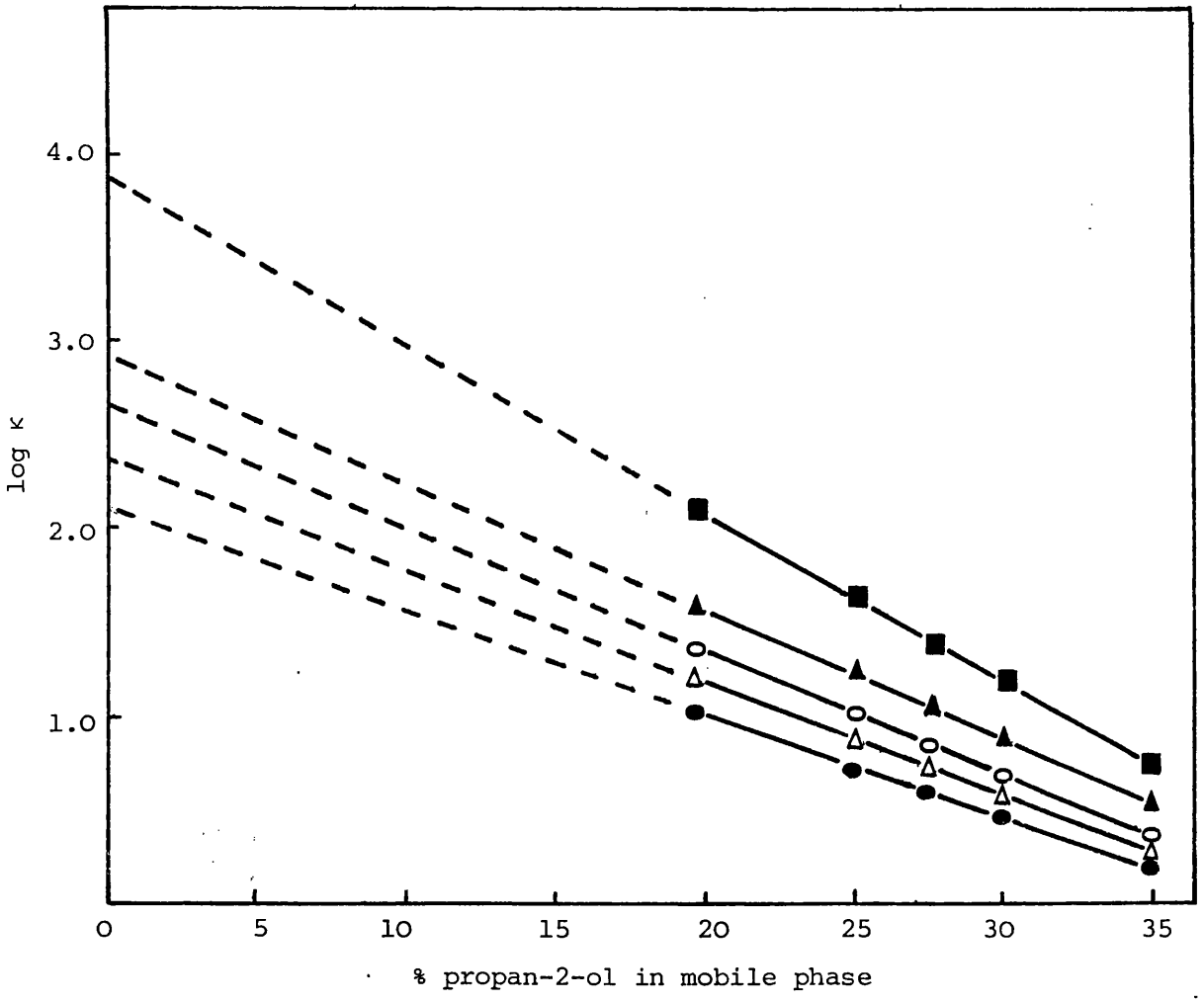


Table 7.7. Data for the regression analysis of the plots shown in Figures 7.5 and 7.6.

Log κ against 1/T

Benzodiazepine	Slope	Intercept	Correlation coefficient
Chlordiazepoxide	1223.6	-2.98	0.9999
Temazepam	1306.5	-3.38	0.9956
Diazepam	1431.0	-3.51	0.9899
Flurazepam	1038.0	-2.05	0.9859
Medazepam	1694.0	-3.68	0.9928

Log κ against % propan-2-ol

Benzodiazepine	Slope	Intercept	Correlation coefficient
Chlordiazepoxide	-0.062	2.42	0.977
Temazepam	-0.055	2.12	0.999
Diazepam	-0.064	2.68	0.998
Flurazepam	-0.065	2.92	0.999
Medazepam	-0.085	3.88	0.999

and at 0% organic modifier (i.e. as would be found in transformation media). The derived κ values are shown in Table 7.8.

Table 7.8. Column capacity ratios (κ) predicted for the 1,4-benzodiazepines on the LiChrosorb RP-2 HPLC column at 27°C, and with 0% organic modifier in the mobile phase.

Benzodiazepine	log κ values at 27°C	log κ values at 0% organic modifier
Temazepam	0.96	2.25
Chlordiazepoxide	1.09	2.55
Diazepam	1.26	2.87
Flurazepam	1.40	3.12
Medazepam	1.95	3.97

The implication of these results is discussed on page 301.

7.5 Discussion

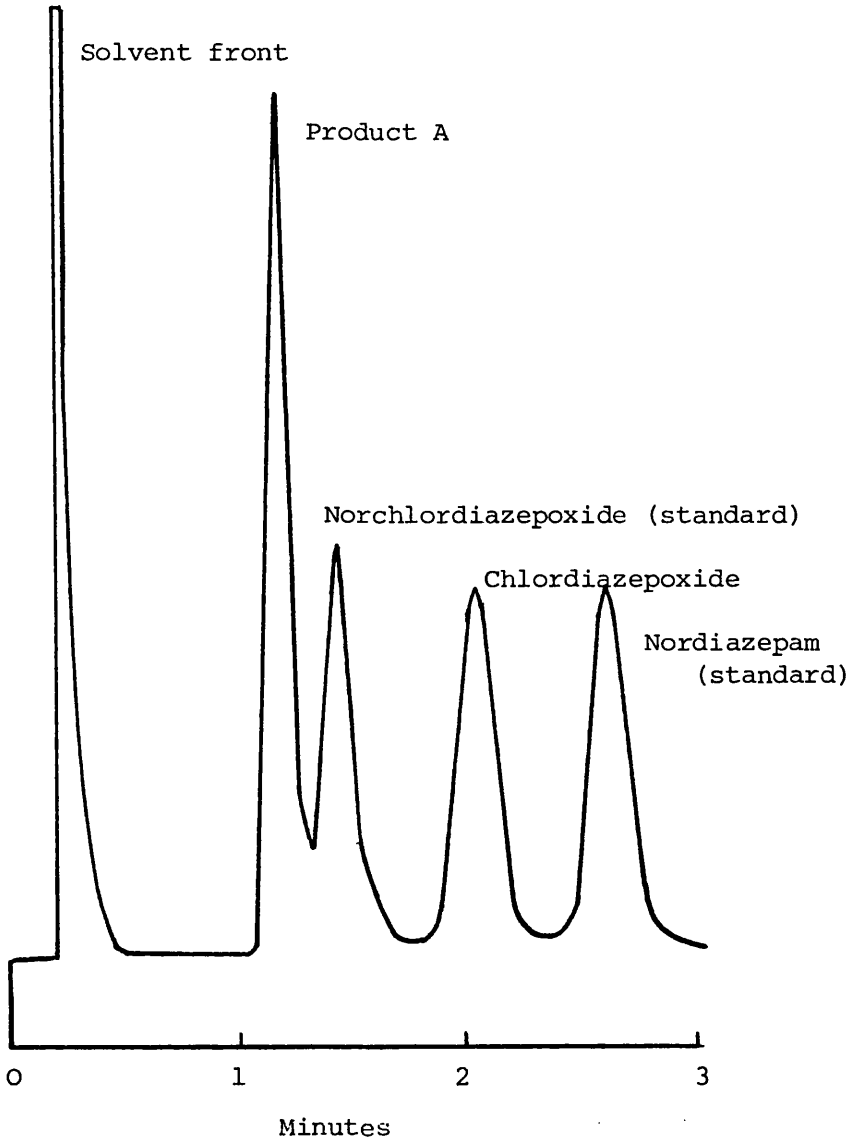
The data in Table 7.6 show that all the 1,4-benzodiazepines studied were transformed by *C. bainieri* to give products which were not detected in the control flasks. The growth of the cultures was not significantly affected by the presence of the test substrates, with the chlordiazepoxide cultures attaining the greatest final biomass. Temazepam (2), diazepam (3) and medazepam (5) each gave single transformation products, and HPLC analysis showed that these products corresponded to the respective normethyl-derivatives. The yields of nortemazepam, nordiazepam and normedazepam were 31.4 μM, 130.1 μM and 261.5 μM respectively.

Chlordiazepoxide (1) also gave a single transformation product (A) in an exceptionally high yield (772.9 μM; Table 7.6). However, HPLC analysis revealed that product A was not normethylchlordiazepoxide. When pure standards of normethylchlordiazepoxide and nordiazepam (also a possible transformation product of 1) were added to a 14-day sample of the chlordiazepoxide transformation mixture, and analysed by HPLC, the chromatogram obtained (see Figure 7.7) clearly shows the retentions of product A and the standards are not the same.

The metabolism of chlordiazepoxide has been reported in man and dog[173-177], but not by micro-organisms. The chlordiazepoxide biotransformation products identified in the plasma of man and dog[178] include, normethylchlordiazepoxide, demoxepam, normethyldiazepam and oxazepam. Structures of these metabolites, and a postulated biotransformation pathway[178] for the metabolism of chlordiazepoxide to oxazepam, in man and dog, are shown in Figure 7.8. Since the HPLC analysis showed that product A did not correspond to the normethylchlorodiazepoxide or nordiazepam standards, the other metabolites in Figure 7.6 were examined. Oxazepam was chromatographed by the chlordiazepoxide HPLC assay procedure but it was found to elute immediately after the solvent front, therefore discounting it as being product A. Unfortunately, demoxepam could not be analysed because there was no standard available.

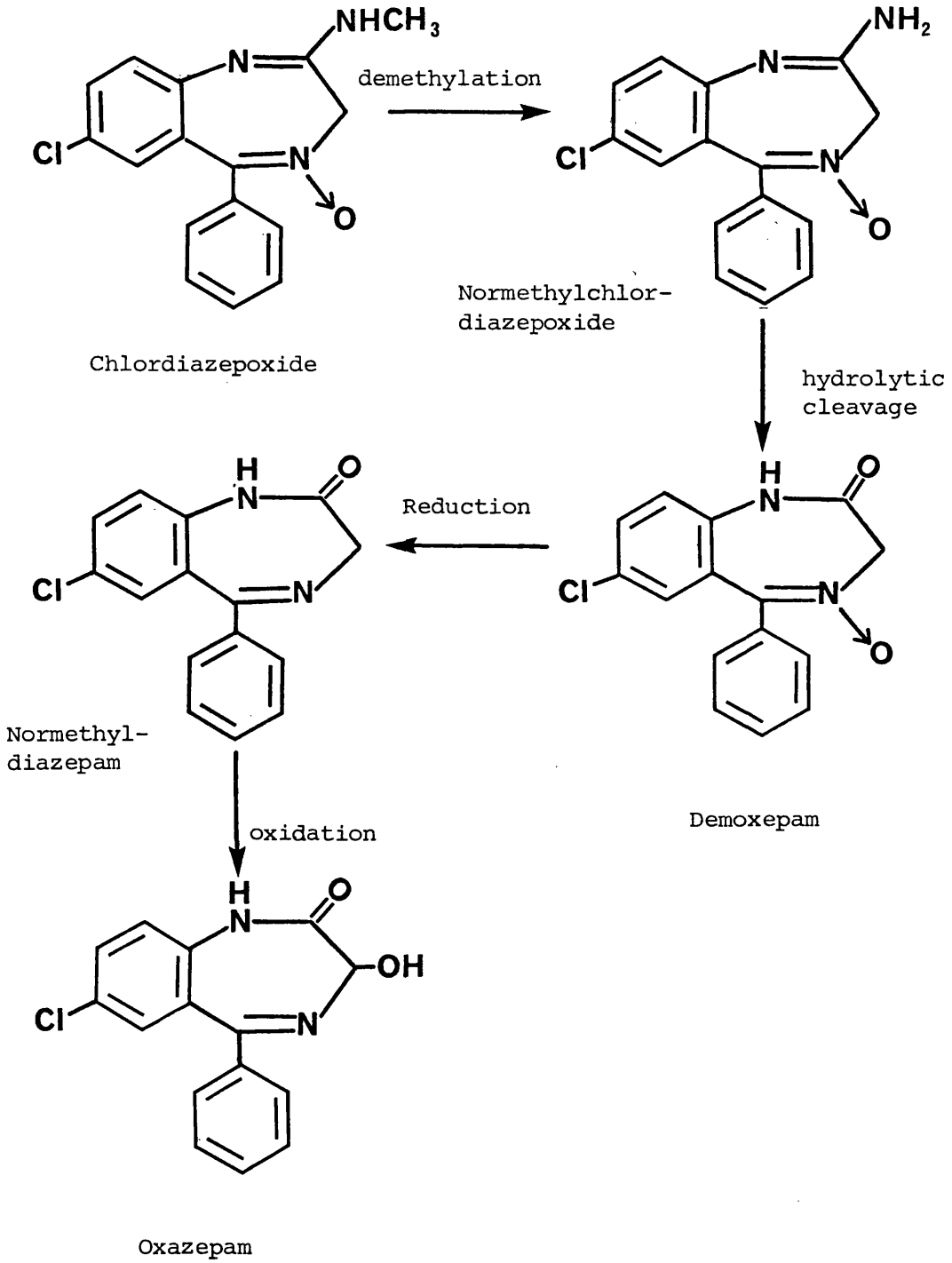
Further attempts were made to identify product A by isolating

Figure 7.7. HPLC chromatogram of a sample taken from chlordiazepoxide transformation mixture, after 14 days incubation with *C. bainieri* at 27°C,250 rpm with the addition of norchlordiazepoxide and nordiazepam standards.



(HPLC conditions as described in Section 7.3.1)

Figure 7.8. Metabolic pathway for chlordiazepoxide to oxazepam in man and dog postulated by Schwartz *et al.*[178]



it from 14-day chlordiazepoxide transformation mixtures. The culture flasks were basified and extracted with chloroform, and an orange solid was recovered from the extracts which HPLC analysis showed to be a mixture of product A (70%) and chlordiazepoxide (30%). This was dissolved in the minimum of ethylacetate, and applied to preparative TLC plates (Silica gel GF) as narrow bands. The plates were developed with ethylacetate mobile phase, and visualised by UV at 254 nm to show that chlordiazepoxide (Rf = 0.13) and product A (Rf = 0.36) were well separated. Product A was scraped off the plates with the silica, and taken up in chloroform. Undissolved silica was removed by filtration (Whatman No. 1) and the solvent evaporated to yield an orange coloured solid. However, HPLC analysis revealed the presence of several peaks as well as product A, suggesting that product A had degraded during the recovery process.

As the chlordiazepoxide-product A mixture obtained from the chloroform extracts showed no signs of degradation, samples were subjected to TLC, NMR and mass spectral analysis. The TLC system developed by Dixon *et al.*[177] to separate chlordiazepoxide from its metabolites was employed. This consisted of silica gel GF plates, chloroform (80); acetone (5); ethanol (5) mobile phase and visualisation by UV at 254 nm. Comparison of Dixon's[177] results with those obtained for the chlordiazepoxide-product A mixture is shown below.

Compound	Dixon's[177] results Rf	Product A mixture Rf
Norchlordiazepoxide	0.1	0.11
Chlordiazepoxide	0.2	0.21
Demoxepam	0.3	No standard
Nordiazepam	0.4	0.38
Chlordiazepoxide-product A mixture	-	0.19, 0.24

An Rf value of 0.24 for product A compared to a theoretical value of 0.3 for demoxepam[177] suggests they were not the same, but this could not be confirmed without using an authentic demoxepam standard.

^{13}C NMR spectra constructed for the chlordiazepoxide standard were compared with spectra constructed for the product A-chlordiazepoxide mixture. Only one additional peak was observed in the spectrum for the mixture, and this occurred at 67.96 ppm relative to TMS (0 ppm), which was a triplet in the off-resonance spectrum. Carbon-13 NMR signal assignments have been made for several benzodiazepines compounds including chlordiazepoxide and its metabolites[179]. The carbonyl group of demoxepam would be expected to produce a signal at approximately 169 - 170 ppm downfield from TMS, but no such peak was observed in the spectrum of the product A mixture. This was further evidence to suggest that Product A was not demoxepam. The chromatographic data (HPLC and TLC) for chlordiazepoxide transformation showed product A, but no additional product peaks, so it

may be inferred that product A was produced directly from chlordiazepoxide. Furthermore, the triplet at 67.96 ppm in the NMR spectrum of the product A mixture implicates the presence of an additional methylene group in product A. This value is similar to those obtained for the carbons of typical N-(hydroxymethyl) groups reported in the previous chapter (page 260), and so product A may be in fact the semi-stable carbinolamine of chlordiazepoxide. Unlike the carbinolamine intermediate of codeine, some stability may be rendered, to the chloridazepoxide carbinolamine by the formation of hydrogen bonds as illustrated in Figure 7.9.

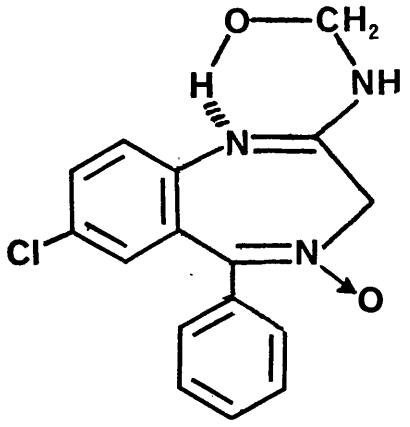


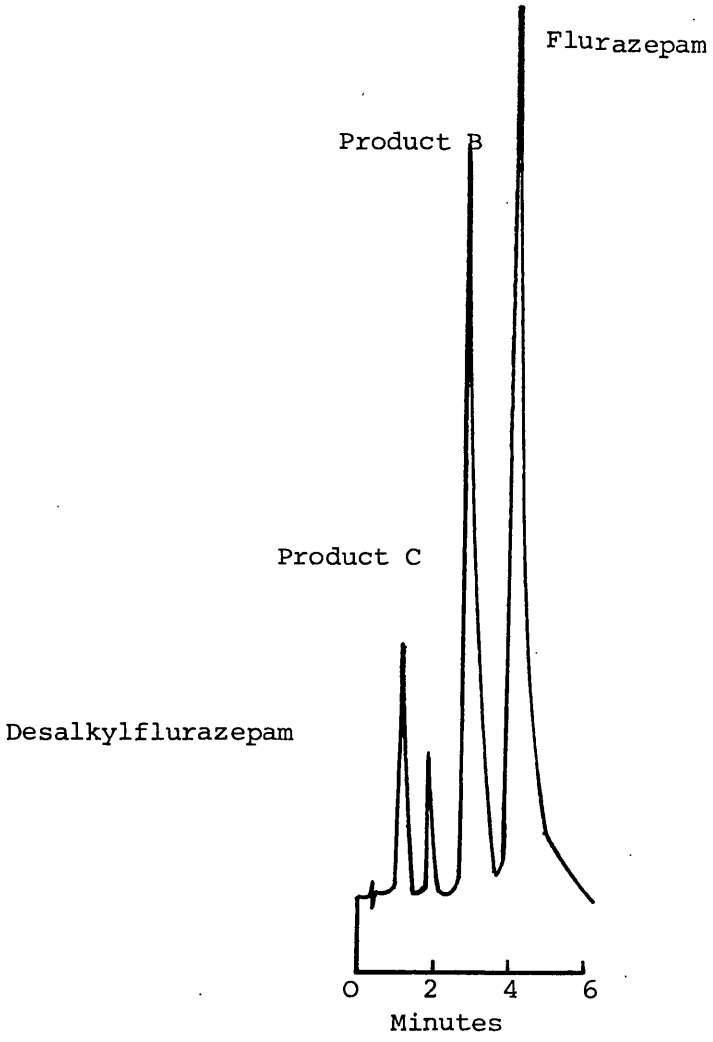
Figure 7.9. The carbinolamine of chlordiazepoxide stabilised by hydrogen bonds

If this was the case, mass spectral data would be expected to show molecular ion fragments of mass 316 corresponding to the carbinolamines, and mass 299 corresponding to chlordiazepoxide also present in the mixture. However, a m/e signal of 316 was not observed, and the fragmentation pattern of the product A-chlordiazepoxide

mixture was almost identical to that of the chlordiazepoxide standard. Although these results proved negative for the carbinolamine, this could be due to its rapid decomposition during the mass spectral process. Overall, no positive confirmatory evidence could be obtained for the identification of product A. Interpretation of these results was made difficult by the instability of product A and also of chlordiazepoxide. The low stability of chlordiazepoxide is well documented[180,181], and it has been reported to hydrolyse to demoxepam in aqueous media under certain conditions. However, there was no evidence of significant chlordiazepoxide degradation in the control flasks in the present study, except after prolonged periods of incubation (12 days or more). It could be possible that chlordiazepoxide hydrolysis was accelerated in the presence of the microorganism, and product A was demoxepam, but this could only be confirmed by acquiring the demoxepam standard and conducting further control experiments.

Flurazepam (4) was transformed by *C. bainieri* to give three products which were not present in the control flasks (see Table 7.6). One of the products corresponded to the desalkylflurazepam standard, but products B and C were not positively identified because of a lack of authentic standards. The incubation was allowed to continue up to 25 days because there was a significant increase in transformation activity from 12 days onwards. A typical HPLC chromatogram of the flurazepam transformation mixture after 20 days incubation is shown in Figure 7.10. Providing the mechanism of HPLC retention was the same for flurazepam and its products these results indicate that products B and C are of intermediate molecular

Figure 7.10. HPLC chromatogram of flurazepam transformation mixture after 20 days incubation with *C. bainieri* at 27°C, 250 rpm.



(For details of HPLC see Section 7.3.1)

size to flurazepam and desalkylflurazepam, and suggest that they have arisen from the loss of part of the alkylamino side chain. The most likely route of flurazepam bictransformation by *C. bainieri* is shown in Figure 7.11

The flurazepam HPLC assay incorporated a pairing ion, pentane sulphonic acid, which reacts with basic nitrogens to form ion pairs depending on the pKa of the solute, and the pH of the mobile phase. At pH 6.4, the nitrogen in the side chain of flurazepam (pKa 7.9)[182], and the nitrogen at position 1 of the diazepine ring of desalkylflurazepam (pKa 11.76)[182] would be expected to form ion pairs with pentane sulphonic acid. Similarly, the metabolites of flurazepam, monodesethyl and didesethyl flurazepam, (Figure 7.11) would also form ion pairs at the nitrogens in their respective alkyl amino side chains. The nitrogen at position 4 in these compounds would be insufficiently protonated at pH 6.4 for ion pairing to occur (pKa flurazepam 1.42, desalkylflurazepam 2.57)[182]. Therefore, it can be assumed that the compounds in Figure 7.11 are retained on the HPLC column in order of molecular weight, and the unaccounted products B and C could be tentatively identified as monodesethyl and didesethylflurazepam respectively. Furthermore the removal of all or part of the alkylamino chain from flurazepam leaves the UV absorbing part of the molecule intact. Since a UV detector was employed, the response factor of the compounds studied (defined as the peak height or area per weight of solute) will be similar, and directly proportional to the retention time of the solutes on the HPLC column. Response factors for flurazepam and desalkylflurazepam were determined directly from the peak

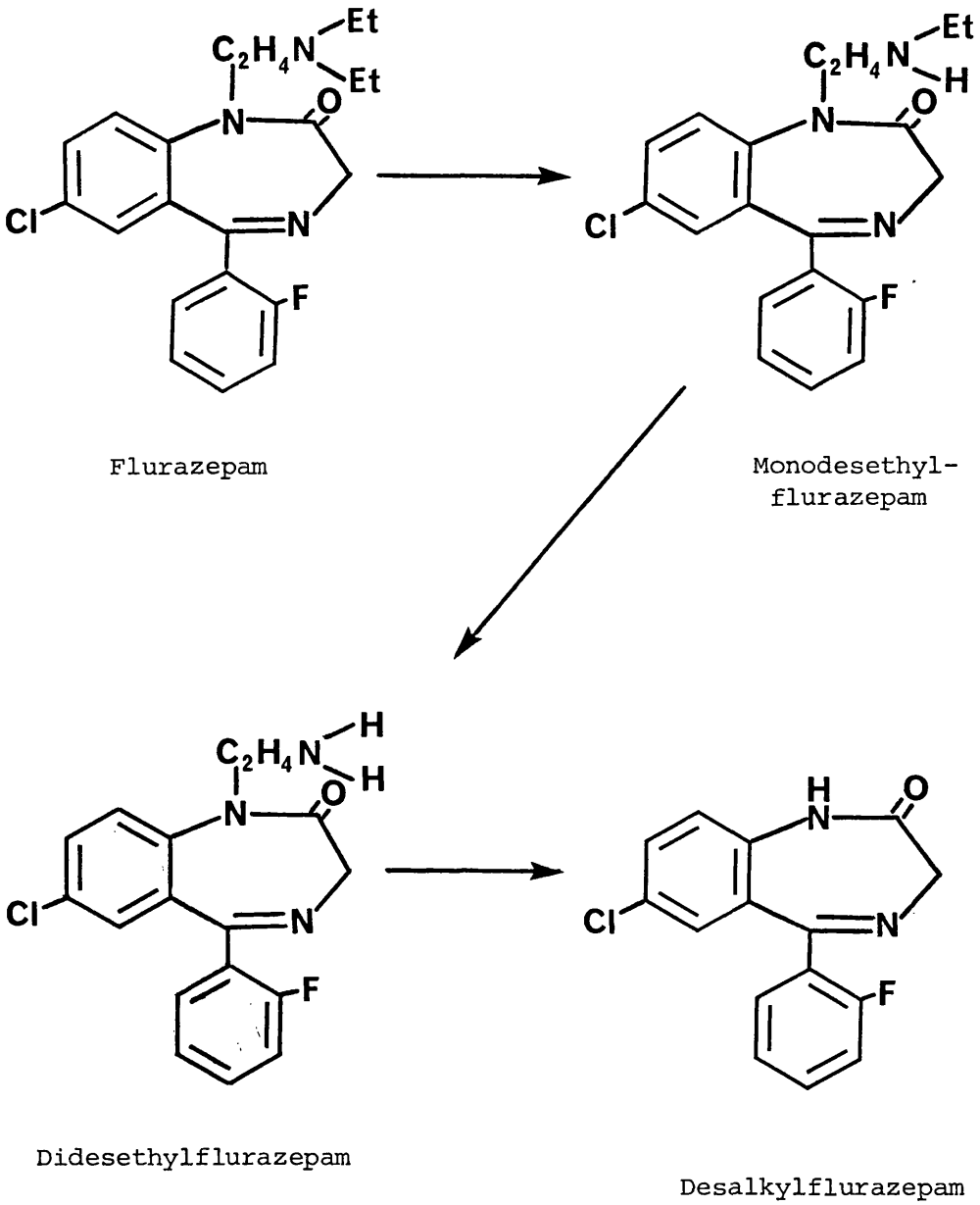


Figure 7.11. Possible route of flurazepam biotransformation by *C. bainieri*.

heights obtained by chromatographing authentic standards of known weight per ml. These response factors were plotted against their respective retention times, and using the known retention times of products B and C response factor values were also obtained from the plot. Equation 7.4 below was then employed to estimate the concentrations of the unknown products B and C in the transformation mixture:-

$$\text{Unknown Product concentration (weight/ml)} \quad (U) = \frac{\text{peak height (U)}}{\text{peak height (S)}} \times \frac{\text{response factor (U)}}{\text{response factor (S)}} \times \text{known conc. (S)} \qquad 7.4$$

The results obtained were used to construct the progress curves for flurazepam transformation by *C. bainieri*, illustrated in Figure 7.4(b). Product B was detected in the transformation mixture after, only three days of incubation, providing further evidence that it could be the monodesethyl metabolite of flurazepam. Product C and desalkyl flurazepam were not detected until much later (day 10-15), This would also be expected if product C was the didesethyl-metabolite, and the flurazepam was metabolised by *C. bainieri* according to the pathway described in Figure 7.11. Although these results were not conclusive because of a lack of authentic standards, the fact that desalkylflurazepam was identified as a transformation product implies that *C. bainieri* was capable of cleaving the whole alkylamino chain of flurazepam. This is an interesting observation considering Sewell's[18] earlier findings that no function greater than methyl was cleaved from a series of 6,7-benzomorphans by *C. echinulata*.. However, further analysis of the transformation products of flurazepam (e.g. Mass spec. NMR etc.) are required to confirm these observations.

Previous experiments conducted by Sewell[18] demonstrated that codeine transformation by *C. echinulata* occurred predominantly within the fungal cells when a codeine substrate was added to the stage-two medium filtrate which had been removed from the mycelium after various stages of incubation, the codeine transformation obtained was found to be insignificant.[18] In Chapter 4 of this thesis it was concluded that the N-demethylase enzyme in *C. bainieri* was membrane-bound. Certainly it is clear that the substrate must penetrate at least one membrane before it is in contact with the enzyme. Because of the lipoid nature of cell membranes in general, it is believed[164] that the greater the lipophilicity of the drug (a high lipid partition coefficient), the faster will be its rate of penetration into the membranes, for subsequent transformation by the enzyme. For a weak electrolyte the partition will be a function of the 'state' of ionization of the substrate[163]; generally cell membranes are more permeable to the unionized form than the ionized form of the drug, mainly because of the greater lipid solubility of the unionized form. Fujita and coworkers[185] have taken into account the ionization of the drug in partition by multiplying the partition coefficient by a factor $(1-\alpha)$, where α is the degree of ionization in the aqueous phase. The benzodiazepines studied are weakly basic compounds with the following reported pKa values[183-184]; chlordiazepoxide 4.6, temazepam 1.31, diazepam 3.3, flurazepam 1.42 and 7.9 and medazepam 4.4. Substituting these pKa values and the pH of the transformation (7.0) into Equation 2.7 (page 59) showed that all the benzodiazepines will be at least 99.9% unionized in the transformation media.

Therefore, α in the factor (1-α) will be insignificant for these benzodiazepines, and their partition coefficients alone can be regarded as a measure of lipid solubility.

The data in Figures 7.5 and 7.6 demonstrate that the column capacity ratios (κ) of the benzodiazepines studied were directly related to the temperature and the % of organic modifier in the mobile phase respectively. Also, the order of retention was constant in each case. Therefore, from the plots it was possible to predict theoretical κ values for a transformation temperature of 27°C, and for a purely aqueous system (Table 7.7). The κ value has been shown to be directly related to the partition coefficient P by Equation 7.3, and so the κ values in Table 7.7 represent the relative lipid solubility of the benzodiazepine compounds. For the substrates temazepam, diazepam and medazepam a direct correlation was found between the κ values (and hence the partition and lipid solubility) and the extent of their N-demethylation by *C. bainieri* (Figure 7.12). It would have been desirable to have more data in support of this evidence, but unfortunately there were no other compounds available for study. As expected, chlordiazepoxide, a secondary amine, and flurazepam, with its long N-alkyl substituent, did not fit the correlation.

The relationship in Figure 7.12 provides evidence that N-demethylation by *C. bainieri* was related to the partition coefficient, a property of the drug molecule as a whole. Temazepam, diazepam an medazepam differ only in the functional groups attached to diazepine ring. It was possible to estimate the contribution of particular groups

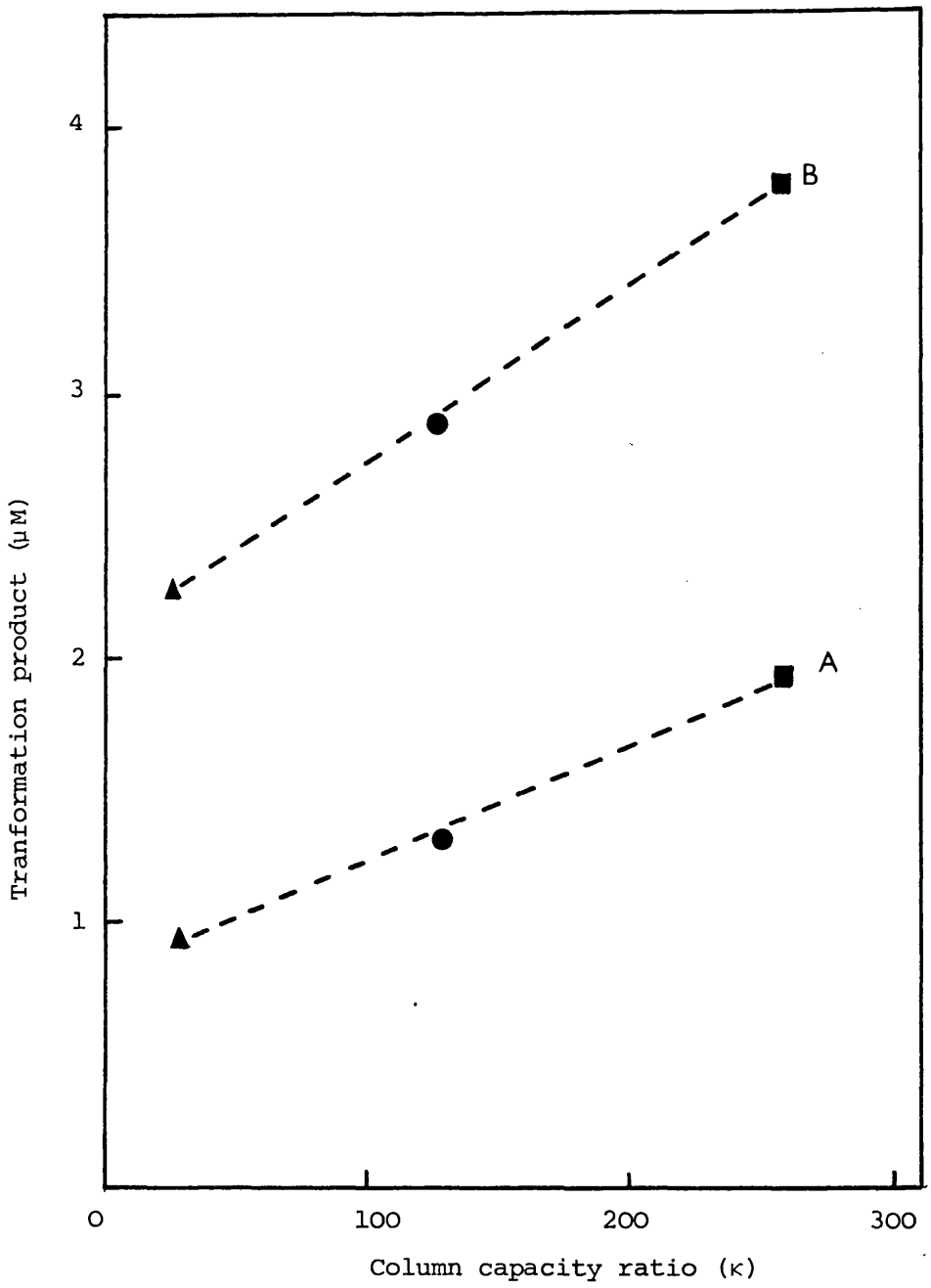


Figure 7.12. Relationship between N-demethylation yield of temazepam ▲ , diazepam ● and medazepam ■ by *C. bainieri* at pH 7.0, and column capacity ratio (κ) obtained at 27°C (A) and 0% organic modifier (B).

or substituents to the overall P value from the chromatographic data[186]. Functional group contributions towards retention was defined earlier in Equation 1.12 as $\tau = \log (\kappa_j / \kappa_i)$ where κ_i is column capacity ratio of the unsubstituted or parent solute and κ_j is the column capacity ratio for the substituted solute. Substituting the retention data for temazepam, diazepam and medazepam into Equation 1.12 gave τ values of -0.69 for the C_2 carbonyl group (diazepam compared to medazepam) and -0.99 for the further addition of the C_3 hydroxyl group (temazepam compared to medazepam). The difference in these values represents the contribution of the hydroxyl group (-0.30) which is similar to the values of -0.3 and -0.45 for 3-hydroxy and 4-hydroxy substituted benzoic acids respectively[193]. The ranges of published τ values depends upon the chromatographic conditions used, especially the percentage carbon loading of the column packing material, so that values are most useful for a given solute series under identical chromatographic conditions. However, even with such a small series of solutes the principle of this approach is clearly demonstrated, and the compilation of further values would be of benefit as indices of hydrophobicity for relating the physico-chemical properties of 1,4-benzodiazepines to their partition (retention, κ) and probable transformation yields.

CHAPTER EIGHT

CONCLUDING SUMMARY AND FINAL DISCUSSION.

CHAPTER 8

8.1 Concluding summary

Previous workers at Bath[18] have demonstrated that several species of the fungus *Cunninghamella* are capable of N-demethylating codeine and other drug molecules containing N-methyl functions. This reaction, which may be difficult to achieve by chemical methods, is often used in the synthesis of drug intermediates. The aim of this project was to make further developments in this field, and in particular, to apply analytical techniques such as HPLC, UV and NMR to overcome some of the problems encountered in transformation studies.

Initial investigations were concerned with the development of HPLC methods for the routine analysis of transformation mixtures. This was to replace the time consuming GLC procedures employed by Sewell[18], which required extraction and derivatisation of the samples. Both straight phase and reversed phase HPLC procedures were developed for the separation and quantitative determination of a model compound, codeine, and its N-demethylated transformation product, norcodeine. The straight phase HPLC separation on a Partisil 5 μm silica column (A) was initially achieved by TLC to reduce time and solvent usage. This procedure was justified by demonstrating that there was a direct correlation between the TLC retention of codeine and its congeners, and their HPLC retention (Figure 2.16, page 114). The straight phase Partisil 5 μm HPLC assay of codeine and norcodeine produced excellent

linear regression results (Table 2.6), but suffered from the disadvantage that the drugs still had to be extracted from the transformation media prior to analysis. This problem was partially overcome by employing a Partisil-10-ODS reversed phase HPLC column (B) to analyse codeine and norcodeine in the transformation media directly. It was not possible to use an internal standard procedure because the large solvent front of the transformation samples tended to obliterate the standard peak. Instead an external standard procedure was employed. A more sensitive HPLC assay employing an ODS-Hypersil 5 μm column (C) was developed for enzyme kinetic studies. An important modification to this assay was the inclusion of a pairing ion, pentane sulphonic acid, to enhance the retentions of the solutes, avoiding any interferences from the solvent front.

The recovery and isolation of codeine and norcodeine from 7 litre batch cultures of transformation media was achieved by a two-stage procedure. In the first stage, various non-ionic resins and reversed phase materials were investigated to determine the optimum conditions for the adsorption and desorption of the drugs from fermentation media prior to their separation, by preparative HPLC. Amberlite XAD-4 resin was found to be the most suitable, and a column containing 100 g of resin was capable of extracting up to 100 mg of combined codeine and norcodeine in 250 ml of transformation media with extraction efficiencies of 85.8% and 98.5% respectively. In the second state of the extraction procedure, straight phase and reversed phase modes of preparative HPLC were compared for their loading and separation capabilities. The reversed phase method provided a superior throughput and loading capacity

which was only limited by the solubility of the drugs in the mobile phase. However, a disadvantage of this procedure was the necessity for a further extraction step to recover the drugs from the aqueous mobile phase. Overall it would probably be more economical in long term use compared to the straight phase system, as it employs a relatively simple and cheap mobile phase, and was found in practice to provide longer service without the need for regeneration or repacking.

In growth media development studies *Cunninghmaella bainieri* (C43) demonstrated the ability to grow satisfactorily in chemically defined media containing glucose, succinate or tetradecane carbon sources. Over the initial 6-7 days of incubation the rate of growth was similar on all carbon sources, but after this period the glucose cultures easily attained the highest biomass. The reduction in growth rate of tetradecane cultures after 8-10 days incubation was attributed to cellular metabolism of lipids as the carbon source was depleted from the medium. Furthermore, cultures grown on glucose exhibited significant codeine N-demethylation only after the carbon source in the medium was depleted. This was thought to be due to catabolite repression, and was not evident with cultures grown on tetradecane. A possible explanation for this was the rate limiting mass transfer of alkane in to the aqueous media. High specific transformations obtained with tetradecane cultures compared to glucose cultures suggested that tetradecane was a good inducer of the N-demethylase in *C. bainieri*. Increasing the codeine concentrations in the transformation mixture above 0·2 mM significantly reduced the level of codeine N-demethylation

for cultures grown on all the carbon sources. However, the microbial growth appeared to be unaffected by codeine phosphate concentrations up to 8 mM, therefore this observation was attributed to substrate inhibition of the N-demethylase by codeine.

A satisfactory procedure was developed to facilitate the preparation of a cell-free extract from *Cunninghamella* with a degree of N-demethylase activity far superior to that obtained by Sewell's original method[18]. The requirement of a surfactant, Triton X100, in the extraction procedure, suggested that the N-demethylase was membrane bound. However, the N-demethylase activity of extracts decreased with Triton X100 concentrations above 0.5% v/v, and a concentration of 0.33% v/v was found to be optimal. Further attempts to enhance the N-demethylase activity of the cell-free extract by dialysis, or by a calcium chloride precipitation technique, proved unsuccessful or impracticable. It was demonstrated that formaldehyde generated from the incubation of codeine with cell-free extracts arose from N-demethylation of the codeine substrate. The amount of codeine N-demethylated by cell-free extracts of *C. bainieri* increased with codeine concentration up to a concentration of 3 mM in the incubation mixture. The activity of cell-free extracts was also increased independently by the addition of the co-factors Fe^{2+}, NADH and NADPH. However, there was no preference of the system for NADPH rather than NADH, but there was a perceptible synergistic effect observed by adding NADPH and NADH together. It was demonstrated that codeine N-demethylation was limited by the availability of endogenous cofactors utilised in the reaction by *C. bainieri* and not replenished.

Cell-free extracts stored at 4°C, in a nitrogen atmosphere, retained a satisfactory degree of N-demethylase activity over the period of time required for subsequent studies. The presence of a maximum absorbance in the 450 nm region of the UV carbon-monoxide reduced difference spectrum provided strong evidence that the N-demethylase from *C. bainieri* was a Cytochrome type P-450 mono-oxygenase. Enzyme activity of cell-free extracts from *C. bainieri* was estimated in terms of codeine N-demethylation using the ODS-Hypersil HPLC assay to determine the transformation product, norcodeine. This technique was used to determine apparent Michaelis constants (K_M) and maximum velocity ($\underline{V}$) values for the N-demethylation of codeine and codeine N-oxide substrates. Data from the kinetic studies implied that codeine N-oxide was not an intermediate in the N-demethylation of codeine by *C. bainieri*. Further evidence to substantiate this view was obtained from investigations on the effect of selected enzyme inducers and enzyme inhibitors on the same transformations. Codeine N-oxide was found to be a less effective inducer than codeine phosphate or codeine base. Codeine N-demethylation was inhibited by carbon monoxide and SKF 525A typical of cytochrome P-450. However, potassium cyanide produced no significant inhibition of codeine or codeine N-oxide N-demethylation, and codeine, as well as norcodeine, was detected in the codeine N-oxide reaction mixture. On the basis of these data it was proposed that codeine N-demethylation by *C. bainieri* occurred via α-C oxidation rather than N-oxidation, to produce a carbinolamine intermediate. However, this would be difficult to confirm as the carbinolamine is unstable, and hence transient, decomposing to norcodeine and formaldehyde.

An elaborate ^{13}C spectroscopic technique was developed to detect the possible intermediates in the N-demethylation of codeine. Codeine $\left(N\text{-}Me\text{-}^{13}C\right)$ HCl was N-demethylated in the NMR tube by cell-free extracts of *C. bainieri* to give norcodeine and ^{13}C labelled formaldehyde. The labelled formaldehyde liberated was trapped with sodium sulphite and the sulphite adduct, as well as intermediates were located in the NMR spectrum. Codeine N-demethylation was observed at a variety of selected temperatures from 5 to 40°C. Intermediate resonances assignable to codeine N-oxide were not detected in any of these enzyme transformation studies, and a methine signal was not observed in the OFR spectrum. However, a resonance signal was observed, occurring at all the temperatures studied, which corresponded to a possible intermediate. This signal was a triplet in the OFR spectrum implicating a methylene group (CH_2) and was tentatively identified as the N-(hydroxymethyl) group of a possible codeine carbinolamine intermediate. These results were consistent with the previous kinetic data, and provided further evidence regarding the mechanism of codeine N-demethylation by *C. bainieri*.

The dependence of microbial N-dealkylation on substrate lipophilicity was examined by determining the column capacity ratios (κ) of a selected group of 1,4-benzodiazepines, and plotting these values against the microbial transformation yields obtained over a fixed incubation period with growing cells of *C. bainieri*. However, chlordiazepoxide was not N-demethylated, but a transformation product was observed in high yield which was

tentatively identified as a carbinolamine, possibly stabilised by intra-molecular hydrogen bonding. Flurazepam was transformed to various products, including one which corresponded to the desalkylflurazepam reference standard, indicating cleavage of the N-(diethylamino)ethyl function. Of the benzodiazepines with N-methyl functions, temazepam, diazepam and medazepam, N-demethylation was effected in yields of 3%, 12% and 26% respectively . These transformation yields were directly proportional to the predicted κ values (see Figure 7.12, page 302), and suggested that microbial N-dealkylation was affected more readily as the lipophilic character of the substrate was increased.

8.2 Suggestions for future work

The present studies have been mainly concerned with the development of analytical techniques which were applied to study the microbial transformation of a model compound codeine. These techniques should be utilised in future studies to examine alternative transformation systems. For example, to scale up the transformation of a selected benzodiazepine compound, and to employ the Amberlite resin/preparative HPLC extraction procedure with slight modifications to isolate the products. This would be of value in the preparation of reference metabolites, and the laboratory scale preparation of nor-intermediates of drug compounds which may be difficult to obtain by chemical procedures. The ^{13}C NMR spectroscopic technique, described in Chapter 6 of this thesis, should also be employed to detect possible intermediates in the N-dealkylation of alternative

drug molecules. The substrate would have to be ^{13}C enriched, and if not available commercially, would have to be synthesised from its nor-intermediate compound. Again, preparative HPLC could be utilized to isolate enough nor-intermediate from a microbial transformation mixture to do this. Data from these NMR studies may provide a more complete picture concerning the mechanism of microbial N-dealkylation of drugs.

The induction of *Cunninghamella* sp. was shown to be severely catabolite repressed by the presence of carbohydrates, such as glucose, in the medium. Repression was not apparent when a tetradecane carbon source was employed, and the results demonstrated that tetradecane was in fact a good inducer of the N-demethylase enzyme. Therefore, in future experiments, catabolite repression may be avoided by growing the cultures initially on glucose to achieve a high cell-mass quickly and when this has been achieved, the glucose should be made limiting and a tetradecane inducer added. In this way, maintenance energy for the duration of the transformation period will be provided in the form of the alkane.

Codeine N-demethylation by *C. bainieri* was demonstrated to be limited by the availability of endogenous co-factors (NADH, NADPH) in the incubation mixture (Chapter 4, page 183). Although this may be overcome by incorporating a co-factor regeneration system[189] to enhance the transformation it is doubtful if this would be viable on a larger scale of study. For example, it may be

possible to use the formaldehyde liberated from N-demethylation as a potential source of reducing equivalents for co-factor regeneration. A possible alternative solution to this, and to the problems previously discussed, may be to immobilise the microbial cells on an inert support. - Whole cell immobilization may overcome the problem of co-factor provision[188]; and offers further advantages which include the regeneration and reuse of cells; a greatly increased catalyst density, increased cell stability and lower risks of contamination from other microorganisms[188]. It would avoid the requirements of an enzyme extraction procedure, and catabolite repression and substrate inhibition could be avoided. Preliminary studies by Hubble[194] showed that the cells of *C. echinulata* could easily be immobilised onto calcium alginate gel. However, the N-demethylation of codeine phosphate was unsuccessful because the phosphate caused dissolution of the gel entrapment matrix. Other workers with immobilised systems have had more success. For example, Schubert *et al.*[190] managed to entrap an active microsomal P-450 system in calcium alginate which was capable of N-demethylating aminopyrene and ethylmorphine. Also Yamamoto *et al.*[191] immobilised the cells of *Pseudomonas putida* ATCC 4359 on a polyacrylamide gel lattice to provide the continuous production of L-citrulline at 37^{o}C for 1 month. Future studies should investigate the possibility of using alternative support materials for the immobilization of whole cells of *Cunninghamella*. Inert materials such as polyacrylamide gels, collagen dispersions or polymeric metal hydroxide gels could be investigated. A system may be developed for the transformation of the model compound codeine and also for selected benzodiazepines.

It has already been suggested that the ^{13}C NMR studies should be continued with other drug molecules containing ^{13}C enriched N-methyl groups. These data could be supported by transformation studies with substrates labelled with ^{14}C, both in the N-methyl group, and also in the nucleus of the molecule. In this way the fate of the formaldehyde liberated during microbial N-demethylation could be investigated. For example, subsequent analysis of the various cell fractions may reveal the ability of the cell to assimilate formaldehyde from N-dealkylation in a possible detoxification mechanism. It may also be possible to determine the actual site of N-demethylation in the microbial cell with the aid of the radiolabelled precursors.

The results of the benzodiazepine structure-activity study suggested there was a direct relationship between substrate lipophilicity and N-demethylation by *C. bainieri*. Further studies should be performed with other substituted benzodiazepines to confirm this correlation. The structures of compounds suggested for further study are illustrated in Figure 8.1

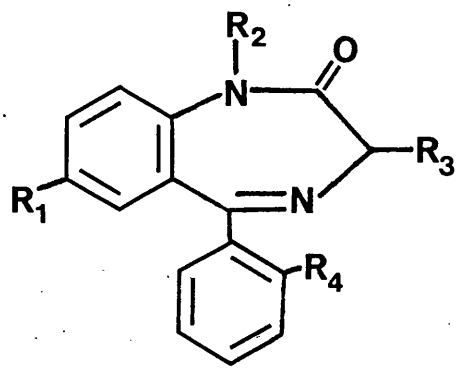
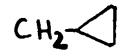


Figure 8.1 Structures of 1,4-benzodiazepines for further study
(For table see page 314)

Figure 8.1 Structures of 1,4-benzodiazepines for further study (cont.)

	R_1	R_2	R_3	R_4
Methylbromazepam	Br	CH_3	Pyridyl	H
Methyl nitrazepam	NO_2	CH_3	H_2	H
Methyl clonazepam	NO_2	CH_3	H_2	Cl
Flunitrazepam	NO_2	CH_3	H_2	F
Prazepam	Cl	CH_2- 	H_2	H

Further studies should be performed to confirm the identity of the chlordiazepoxide transformation product (A) described in Chapter 7. Preparative HPLC could be employed to isolate Product A for further analysis and identification. If product A is the carbinolamine suggested, it should be further investigated for possible cytotoxic properties.

An alternative approach to improve the microbial transformation of polar substrates with ionizable groups might be to react the drug with an ion pair of opposite charge. Since upon ion pairing the ionized substrate tends towards electrical neutrality it will have greater lipoidal solubility. The use of ion pairs to improve the biological absorption of ionized molecules has been proposed by other workers. For example, Tomlinson[192] has shown that the penetration of anhydrocarbonaceous polyamide 6 membrane by sodium cromoglyate was enhanced substantially by the presence of

an organic ion of opposite electrical charge. This idea should be pursued in an attempt to improve the transformation yield of compounds such as codeine phosphate or chlordiazepoxide. HCl. Initial experiments should be concerned with employing a relatively small ion-pair reagent e.g. C_2or C_3, and the effect of the size of the carbon chain of the pairing ion on microbial transformation might then be investigated.

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